

A Human Stem Cell Project?

Advances in the study of embryonic and adult stem cells offer opportunities to boost research on both cell types towards clinical applications. But funding and coordination at national levels will be required to make the most effective progress.

This issue of *Nature* contains two significant papers on stem cells, prompting an assessment of the scientific and political terrains ahead and of some of the hurdles that must be overcome to bring stem-cell therapies to the clinic. One paper, by Catherine Verfaillie and colleagues (see page 41), describes a kind of adult stem cell that may turn out to be as versatile as embryonic stem (ES) cells. The other, by Ron McKay and colleagues (see page 50), shows that mouse ES cells can generate neurons effectively to relieve symptoms of Parkinson's disease in a rat model.

Opponents of ES-cell research are already heralding Verfaillie's adult stem cells (called multipotent adult progenitor cells, or MAPCs) as proof that work on human ES cells is no longer needed. Appropriately, the message from the stem-cell research community has been a resounding call for more research on both adult and ES cells in animal models of human diseases. It is far too early in the game to discount ES cells that can perform as powerfully as those studied by McKay and other researchers. And it is clear that thorough functional characterization of engrafted MAPCs needs to be carried out before it can be said that these cells have the same kind of clinical potential as ES cells.

Stem-cell researchers have already greeted both reports with cautious optimism, but they note that it will be years before human therapies emerge from this and other stem-cell research, and cite gene therapy as an object lesson in the dangers of promising too much, too soon. Verfaillie's findings have been rumoured for over a year, but her results are so remarkable that they await confirmation by many other labs to gain true acceptance. If confirmed, there will need to be a coordinated effort to train researchers to use MAPCs, derive new cell lines, and make them widely available to the scientific community. Interest in the cells will be intense, and the burden of training and distribution should not fall on Verfaillie's modestly scaled academic lab.

A coordinated endeavour

Likewise, to realize the potential of human ES cells, coordinated efforts should be made to standardize techniques and practices for growing and differentiating cells, from all available sources. This will be necessary to determine which ones perform best for different applications, and to develop ways to scale up production and provide a uniform, highly characterized source of human ES cells for primate studies, which will now be needed to confirm the rodent experiments. Putting in place such a cohesive research infrastructure could also provide a transparent and regulated framework so that the derivation of new human ES-cell lines can be conducted according to approved procedures and ethical guidelines.

The research community widely acknowledges that the creation of new ES-cell lines will be necessary to obtain cells with optimal growth and differentiation, and this will no doubt be the case for MAPCs as well. In countries such as Israel, Singapore and Britain, where researchers are permitted to derive ES-cell lines from few-day-old embryos generated by *in vitro* fertilization, deriving new cell lines is a priority.

However, since President George W. Bush's ruling on 9 August 2001, US researchers with federal funding must make do with the

64 ES-cell lines that existed before that date. In fact, the reality seems much more restrictive, as scientists have complained that only a few of these lines are actually available. Moreover, the available lines are said to be difficult to cultivate, and in some cases carry hefty price tags or heavy intellectual-property entanglements.

An example of the kind of analysis that would hasten the progress of work on embryonic and adult stem cells is demonstrated by labs in Israel. Researchers there are painstakingly comparing the characteristics of cells sent to them from around the world, albeit with only a handful of human ES-cell lines at present. This type of effort must be carried out on a much greater scale for both ES cells and MAPCs, if further studies confirm their clinical promise.

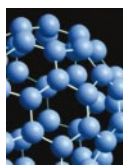
Growth funds

The US National Institutes of Health (NIH) has made some movement in this direction, by providing \$1 million in grants for establishing courses on human ES cells (see <http://grants1.nih.gov/grants/guide/pa-files/PA-02-054.html>). It has also provided \$3.5 million to enhance resource infrastructure at two companies and two universities that between them possess 17 human ES-cell lines that meet Bush's criteria. The grants are meant to promote the expansion, testing, quality assurance and distribution of human ES-cell lines (see <http://www.nih.gov/news/pr/apr2002/od-26.htm>).

ES cells and MAPCs represent tools for defining the molecular basis of pluripotency (the ability to form most or all tissues or cells of an organism), as well as the molecular cues that steer these 'blank' cells to different developmental fates. Scientists must now explore how ES cells and MAPCs are alike and how they differ, by growing and analysing them side by side. This should ideally be done at facilities that have demonstrated expertise in working with both cell types, something that doesn't exist at this time. Will the observed differences between MAPCs and ES cells prove to be fundamental to their developmental potential? What other differences will emerge? For example, it seems that MAPCs are good at forming liver cells, but not cardiac cells, which ES cells form easily in culture. Undifferentiated ES cells form tumours when injected into animals — will it be possible to remove potentially contaminating ES cells from differentiated cell populations? Only by making these studies comprehensive and carrying them out under highly standardized and reproducible conditions will meaningful conclusions emerge. In this way, we can discover the appropriate practices, protocols and high-quality cell sources for clinical trials.

Several stem-cell institutes have been initiated worldwide, albeit all on a relatively small scale compared with, for example, the heavily funded and highly coordinated enterprise to sequence the human genome. The \$3.5 million the NIH has earmarked for infrastructure grants seems absurdly small, especially when weighed against the extraordinary promise of stem cells to deliver therapies for many human diseases in a relatively short time. Researchers estimate that it will be about a decade before stem cells can be used to treat human diseases, but by applying the lessons in teamwork and leadership learned from other big-science endeavours, this time line might be accelerated. Perhaps it's time to start thinking about a Human Stem Cell Project. ■





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Fire-fighting senators move to set up wildfire research institutes

Tony Reichhardt, Washington

As almost a million acres of American forest burned out of control last week, senators representing western states proposed setting up three wildfire research institutes to study what is becoming an ecological disaster for their region.

Wayne Allard (Republican, Colorado) and Jon Kyl (Republican, Arizona) introduced legislation on 24 June that would allot \$15 million to create three institutes — one in each of their states and one in New Mexico — to study ecological approaches to the wildfire problem.

If approved, the money would almost double the \$16 million spent this year on a Joint Fire Science Program administered by the US Forest Service, which is part of the Department of Agriculture, and the Department of the Interior. The nationwide programme awards grants to study the wildfire problem, with an emphasis on the natural 'fuels' that feed large forest fires. The new centres would focus specifically on the western United States, although it is unclear how the grant money would be distributed.

Scientists and land managers generally



Arizona's worst forest fire still rages while controversy engulfs the science of forest management.

agree on the underlying causes of the problem. Overgrazing has long since stripped the region of grasses that historically provided the fuel for frequent, but far less destructive, fires. That, combined with

the Forest Service's decades-old policy of extinguishing all forest fires as quickly as possible, has led to a dangerous build-up of dense stands of small trees among the more sparse large trees. It is the small trees that fuel intensely hot wildfires such as the recent ones in Arizona and Colorado.

There is also general agreement that these fuels must be reduced, either through deliberate 'prescribed' burns, or by thinning out the smaller trees, but each solution has drawbacks. Prescribed burns become more difficult as the edge of the forest becomes more populated. They produce smoke, and can themselves get out of control, as happened, for example, during a fire in May 2000 near Los Alamos, New Mexico (see *Nature* 405, 264; 2000).

The alternative approach of thinning the trees, primarily by logging, is the subject of a fierce, perennial debate between environmentalists and foresters in the region. Kyl was among the Republican politicians who blamed the Rodeo-Chediski fire, which began on 18 June and is already Arizona's worst recorded fire, on environmentalists' opposition to logging in national forests. Environmentalists, in turn, have accused timber companies and politicians

Top physics lab names its leader

David Cyranoski, Tokyo

Japan's High Energy Accelerator Research Organization (KEK) has chosen its next director — Yoichi Totsuka, a globally renowned neutrino scientist with wide experience of running large projects.

Totsuka is a professor at the University of Tokyo's Institute for Cosmic Ray Research and heads its Kamioka Observatory, where he has spent recent months rebuilding the Super-Kamiokande (Super-K) neutrino detector after an accident destroyed most of its sensors (see *Nature* 416, 118–119; 2002).

He will take over KEK — Asia's largest and most prestigious high-energy physics laboratory — next April, when Hirotaka Sugawara, its present director, returns to research at the University of Hawaii.

Totsuka is best known for providing evidence that neutrinos 'oscillate', or change from one kind to another (see *Phys. Rev. Lett.* 81, 1562–1567; 1998).

"Super-K has done half of its work," says Totsuka, who this year turned 60, the mandatory retirement age for professors at the University of Tokyo. "I wish I could do more, but there is a time limit."

Totsuka was the only nominee put forward by KEK's board of councillors to the science minister, who must still confirm the appointment. But the committee's recommendation has never been rejected.

Sugawara says that he will be advising Totsuka on the challenges ahead of him. "It will be the most active high-energy physics lab in the world," predicts Sugawara. ■

of using the fires as an excuse to gain access to larger trees that are more commercially valuable, but are not part of the wildfire problem.

Meanwhile, the west is bracing itself for what could be its worst fire season in decades. Already, 2.8 million acres nationwide have burned, compared with 1.6 million in the first half of 2000, which ended with 8.4 million acres destroyed, the most in 40 years. The problem gets worse each year, with more suburban encroachment into forested areas complicating efforts to contain fires, and increasing their economic and human costs.

Julio Betancourt, a research scientist with the US Geological Survey's Desert Laboratory in Tucson, Arizona, who is studying the ecology of the desert southwest, warns that if the region goes into a prolonged drought lasting years — and climatologists see signs that this may be happening — the situation will become even worse. And fires aren't the end of it, he says. When monsoon rains come to Arizona later this month, as they do every summer, run-off from the now barren landscape is likely to lead to serious erosion and flooding.

Betancourt and other scientists worry that even with more money for research, and a more determined effort to eliminate hazardous fuels, this may be too little, too late. According to a report by the Joint Fire Science Program, it "may be possible" to treat 5 million acres a year, but with more than 100 million acres at risk from fire nationwide, "it is uncertain whether this treatment level is adequate to reverse the trend of increasing fuel accumulations and begin to reduce wildland fire problems". ■

Visitors to AIDS conference trapped in web of red tape

Declan Butler, Paris

A row over the availability of visas for visitors from developing nations is casting a shadow over the 14th International AIDS Conference, which opens in Barcelona on 7 July.

Scientists and journalists from several countries, including India, Colombia and the Democratic Republic of the Congo, say that they have had their visa applications blocked or delayed by local Spanish embassy officials.

Earlier this year, the Spanish foreign ministry contacted its embassies around the world asking them to expedite visas for participants in the conference who meet certain requirements, such as having medical insurance and sufficient money to return home.

But the message failed to get through, according to Lars Kallings, secretary-general of the Stockholm-based International AIDS Society, one of the conference's organizers. Kallings says that visa applications have been hampered by bureaucracy, and that staff at various embassies and consulates have been unhelpful. Some applicants have complained that the entry requirements are excessive, Kallings says, whereas others allege that wealthier tourists have been given priority for the fixed quotas of available visas.

John Mokili, a Congolese postdoc who is currently working at the Los Alamos National Laboratory in New Mexico, says that he has only just received his visa — a month after he applied for it. The process should normally take a day. As of 1 July, he says, only one of 14 other Congolese researchers had their visas.

And of 72 journalists from developing

nations offered scholarships to attend a training programme on reporting on HIV, only 25 have their visas, says Laurie Garrett, a New York-based journalist helping to organize it.

Kallings says that the situation could look as though Spain has breached its contract to host the conference without discrimination. He points out that the United States remains blacklisted as a venue for the conference after similar problems in 1985 and 1987.

Conference organizers have pressed the Spanish government to redress the situation and one organizer, Javier Rubio, says a list of registered participants has now been circulated to Spain's embassies and a fresh effort is under way to speed up visa applications. ■

♦ www.aids2002.com



No entry: delegates from developing nations are finding it hard to gain access to Barcelona.

DALLAS & JOHN HEATON/CORBIS

Gulf War syndrome research poised for cash injection

Jonathan Knight, San Francisco

The US government is set to give research into Gulf War syndrome a sharp increase in funds following advice from scientists who believe that ill veterans suffered neurological damage during the 1991 conflict.

On 25 June, an advisory panel appointed by veterans' affairs secretary Anthony Principi concluded that research into whether neurological damage had been caused by vaccination or exposure to nerve agents "should be aggressively pursued", and recommended that Congress commit \$450 million over three years to the project.

The administration of President Bill Clinton tended to play down the idea that nerve gas or vaccines might have caused the health problems reported by some veterans, calling evidence of any link inconclusive.

Instead, it maintained that the most likely cause was traumatic stress.

But during his election campaign, President George W. Bush promised to investigate evidence of such links, and he has since appointed officials, such as Principi, who are sympathetic to the veterans' claims.

The science of Gulf War syndrome has changed little over the years, however. Epidemiological studies have shown that more Gulf War veterans suffer symptoms such as dizziness compared with other veterans, but a decade of research has failed to find a single 'syndrome', or its cause.

In September 2000, an Institute of Medicine panel reviewed 1,000 research papers and concluded that there was insufficient evidence to support a link between nerve agents and veterans'

symptoms (see *Nature* 407, 121; 2000).

The advisory panel, which included several researchers whose work has pointed to a neurological cause of Gulf War syndrome, is urging that the new money should be administered by the National Institutes of Health. Panel member Robert Haley, director of epidemiology at the University of Texas Southwestern Medical Center at Dallas, says that the defence department cannot be trusted to address the issue fairly. Haley believes that exposure to chemicals may have caused brain damage in some veterans (see *Nature* 385, 187; 1997).

But Gregory Gray, an epidemiologist at the University of Iowa who has studied the problem, says: "A lot of sharp investigators have already looked at this. I'm sceptical that more studies will show anything different." ■

Bell Labs inquiry spreads to superconductors

Geoff Brumfiel, Washington

An investigation into possible data fabrication at Bell Laboratories has been expanded to include three important papers on superconductivity published in *Nature*.

The additions to the investigation, which is being conducted by a panel chaired by Malcolm Beasley of Stanford University in California, have alarmed physicists, dozens of whom have been engaged in efforts to replicate the superconductivity work.

The expansion is also causing trepidation at Bell Labs in Murray Hill, New Jersey — its struggling owner, Lucent Technologies, lost a third of its already-diminished stock-market valuation last week (see graph, right), as investors reacted to news of a multi-billion-dollar fraud at telecommunications firm WorldCom.

Papers co-authored by Jan Hendrik Schön on the use of organic molecules in microelectronics first came under suspicion in May, when researchers noticed two graphs in separate papers that appeared to be identical, right down to the noise in the data (see *Nature* **417**, 367–368; 2002). The Beasley inquiry was set up and soon more than a dozen papers co-authored by Schön were under investigation. Schön stands by his results and is cooperating with the inquiry.

In recent weeks, the inquiry added three more papers co-authored by Schön^{1–3} to its list, Beasley said at the end of last month. The papers describe how two fullerenes — C₆₀ and C₇₀ — and calcium copper oxide lose all electrical resistance (become superconducting) when they have been ‘doped’ using an electric field to add or remove electrons from their crystal structure. Two of the papers^{1,2} were co-authored by Bertram Batlogg, one of the world’s leading experts on superconductor physics, who is now at the Swiss Federal Institute of Technology in Zurich.

The three papers were brought to the panel’s attention by several researchers, including Nobel laureate Philip Anderson of Princeton University, New Jersey, who noticed similarities in the range of temperatures and charges in which two of the materials — C₆₀ and a calcium copper oxide — became superconducting.

Arthur Ramirez, a physicist at Los Alamos National Laboratory in New Mexico, who is trying to reproduce Schön’s work with C₆₀, admits that the similarities are “a little weird” but is withholding judgement on the reasons for them. Ramirez says he will press on with his effort while the Beasley panel completes its evaluation of the original result.

Meanwhile, the troubles of Lucent Technologies continue to mount, with the WorldCom scandal dashing hopes that demand for Lucent’s products will revive

any time soon. “The telecommunications sector is melting down,” says Scott Cleland, head of the Precursor Group, a Washington-based market analyst firm. “Many of these companies, including Lucent, are flirting with bankruptcy.”

David Farber, a professor of telecommunications at the University of Pennsylvania, Philadelphia, and a former researcher at Bell Labs, also sees tough times ahead for the laboratory. “When push comes to shove, what do you sacrifice first? Research,” he says. But

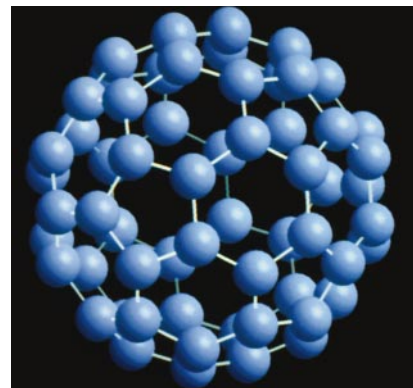
Farber doesn’t believe that the laboratory will shut. Lucent, or whoever takes over its business, will need research to stay competitive in the long run, he says.

Saswato Das, a spokesman for Bell Labs, expressed optimism about the future of basic research there. “We continue to be committed to creating the next-generation technologies to make our company successful,” he says. “Physical sciences research will be an essential component of that.” ■

1. Schön, J. H., Kloc, Ch. & Batlogg, B. *Nature* **408**, 549–552 (2000).
2. Schön, J. H. et al. *Nature* **413**, 831–833 (2001).
3. Schön, J. H. et al. *Nature* **414**, 434–436 (2001).



Downturn: Lucent Technologies’ share price, after stabilizing in 2001, fell sharply last month.



Balls of ire: papers showing superconductivity at 52 K for C₆₀ are being called into question.

Biotech woes set to hit academics

Jonathan Knight, San Francisco

The scandals and stock-market woes that have left the biotechnology industry reeling this year are beginning to take their toll on campuses, according to early indicators.

As biotech stocks collapse — collectively, they have lost two-thirds of their value in the past two years — companies may be shying away from funding research collaborations with academics. That is the picture painted by data from the University of California, San Francisco (UCSF), which has strong links with the state’s powerful biotech sector.

The number of such deals with UCSF peaked at 125 in 2000 for non-clinical research, but then slid to 70 in 2001. In the first 10 months of the 2002 fiscal year, only 39 deals were struck, according to Joel Kirschbaum, director of the university’s technology-transfer office.

Kirschbaum says that investors’ expectations for start-ups have changed. Companies making tools for drug discovery were popular a few years ago, he says, but

investors now want ventures that will rapidly bring a drug or other product to market.

Biotech stocks enjoyed a huge surge in popularity after the dotcom industry collapsed in early 2000. The impending draft sequence of the human genome fuelled the boom, and US biotech firms raised \$38 billion in new capital in 2000, according to the Biotechnology Industry Organization — more than three times that raised in 1999.

But in 2001 new financing dropped to \$15 billion. Now biotechnology is directly implicated in the scandals that have shaken investor confidence in the United States. On 12 June, the former chief executive of biotech firm ImClone Systems in New York, Samuel Waksal, was arrested on charges of insider trading and of alerting family and friends that regulators were about to reject Erbitux, the company’s promising cancer drug.

In this climate, deals to launch new companies are getting harder to broker, says Jonathan Kaufman, science director at LaunchCyte, a company in Pittsburgh, Pennsylvania, that advises entrepreneurs. ■

Recriminations inflame UK research debate

David Adam, London

After a national tour of every university staff room in the country, the debate over Britain's Research Assessment Exercise (RAE) finally rolled into the House of Commons last week, amid acrimony, name-calling and threats of legal action.

Introduced in 1986, the assessment — an exhaustive audit of university research carried out every few years and linked directly to funding — is widely credited with raising the quality of British science, and has attracted interest from governments around the world. But it has not been without controversy, most recently when funds could not be

found to reward record increases in RAE grades awarded by the 2001 exercise (see *Nature* **414**, 834; 2001).

The process has faced even closer scrutiny and much scepticism at home since the results of the latest audit were announced in December last year. On 26 June, the Higher Education Funding Council for England (HEFCE), the body that operates the RAE, announced a major review to decide whether and how the exercise should be repeated. "We want to get people to look forward to what will happen in the future," says Philip Walker, a spokesperson for the council.

But in the Commons debate on 27 June, Ian Gibson, Labour Member of Parliament for Norwich North and chair of the House of Commons Science and Technology Select Committee, said that HEFCE officials had been "arrogant and dismissive" about criticism of the RAE. And a group representing the heads of environmental-science departments wrote to the council on 28 June demanding a review of the way their subject is assessed.

The environmental scientists say that a statistical analysis of the latest audit for their departments reveals irregularities. They say that their departments scored, on average, two grades lower than departments in other disciplines, and complain that there were too few specialists from their subject on the assessing panel. With no appeal mechanism

open to them, some departments have considered asking a court to overturn the results.

"Possible legal action is being discussed but it's expensive and I don't think any university would embark on a legal process on their own," says William Stephens, head of the Institute of Water and the Environment at Cranfield University in Bedfordshire.

Last week the HEFCE also published its response to a highly critical report released by Gibson's committee in April. The committee labelled the assessment a "damaging distraction" and said that it distorts research practices, ruins careers and hastens departmental closures. The HEFCE retorted that there is no hard evidence to support the criticisms. Gibson responded that the council is "out of touch with the community it serves".

Several researchers contacted by *Nature* side with Gibson. One physicist at a leading UK university even claims that scientists' names have been inappropriately added to research papers to boost their departments' scores in the audit.

But HEFCE officials stress that the RAE has achieved its goals, adding that critics are too quick to blame the exercise when unpopular decisions are taken for other reasons. "Disentangling the effects of the RAE from other pressures on the sector is not as straightforward as it may appear," says Bahram Bekhradnia, the HEFCE's director of policy.

CRANFIELD UNIV.



Environmental scientists at Cranfield University and elsewhere feel left out of assessment panels.

Angry scientists march on Moscow in budget protest

Bryon MacWilliams, Moscow

Dozens of scientists trekked more than 130 kilometres over three days, through heavy wind and rain, in a bid to hold Russian President Vladimir Putin to a promise on research spending. It didn't work.

In March, Putin pledged to budget 49.5 billion rubles (US\$1.5 billion) for scientific research in the 2003 fiscal year, which begins in January. But last month, he released a budget proposal that allocates just 35 billion rubles to science.

The long march from Pushchino — a city south of Moscow that houses several large biology laboratories run by the Russian Academy of Sciences — was called by trade unions in protest against scientists' living conditions.

But government officials declined to meet with union officials and said that the revised budget figures would stand. And the march, which began with 30 protesters in the expectation of gathering support along the way, attracted only about 100 scientists. That allowed a closing rally in Moscow on 27 June to be dominated by flag-waving members of



Tired and flagging: beleaguered researchers hold a rally to mark the end of their marathon march.

the opposition Communist Party, who easily outnumbered the marchers.

The latest budget proposal comfortably exceeds this year's annual science spending of 21.7 billion rubles. But union leaders called for the march after meeting with ministers and failing to win promises of more overall spending, better housing allowances for young scientists and

increased grants for graduate students.

"Government bureaucrats are sabotaging the decision of our president to support the sciences," claims Valery Sobolev, chairman of the trade unions of the Russian Academy of Sciences. Union officials say that low wages are forcing researchers to earn money from commercial sources, damaging basic scientific research.

A. ZEMLIANICHENKO/AP PHOTO

US exchange scheme offers a taste of Europe

Quirin Schiermeier, Munich

The flow of young European researchers into the United States may look like a deluge, with a mere trickle in the opposite direction. But the US National Science Foundation (NSF) hopes to address the imbalance through a scheme that will twin its research centres with similar institutions abroad.

The NSF's informal "centre-to-centre" project will encourage the university-based centres funded by the foundation in the United States to identify potential research partners abroad. The NSF hopes that this will encourage young American researchers to venture across the Atlantic.



David Schindel: exchange envoy.

David Schindel, head of the NSF's European office in Paris, announced the scheme at a talk given last month to a group of officials of European research agencies and institutions at the European Commission in Brussels.

On the basis of an e-mail survey of European funding agencies and universities, Schindel is compiling a database of around 600 research centres with structures that are similar to those supported by the foundation in the United States. The NSF has begun to disseminate the information to the heads of 250 US centres, encouraging them to match themselves with appropriate partners.

The NSF-funded centres, which carry out basic research in a variety of disciplines, each receive annual support of between \$500,000 and \$5 million from the foundation. Potential European partners include Germany's International Max Planck Research Schools, the Interdisciplinary Research Collaborations funded by the UK Research Councils, and the Centres of Excellence funded by the European Union in central and eastern Europe.

The NSF's Office of International Science and Engineering will finance travel costs and introductory workshops aimed at initiating contacts, says Rose Gombay, a programme manager for western Europe who is based at the NSF's headquarters in Arlington, Virginia. Further funding will be made available if joint research activities materialize, she says.

The main goal of the new initiative is to encourage postdoctoral fellows and PhD candidates in the United States to spend some time overseas, says Schindel.

"There is no doubt that transatlantic traffic of young researchers is primarily one-way," Schindel explains. "We would like many more young US researchers to travel to and work in Europe and elsewhere."

The number of young American researchers working in Europe is low — there

are fewer than 300 US postdocs in Germany, for example. On the other hand, 40% of the 20,000 postdocs in the United States are of non-US origin.

The NSF acknowledges the lack of mobility of young scientists in the United States. "There is a strong cultural barrier, and there is the sheer size of the US system that feeds the belief that it is not necessary to go abroad," says Mark Suskin, a programme manager at the NSF's international office. "However, international experience is indispensable for today's scientific workforce."

Fostering exchanges for young researchers



is already a priority for the NSF's international office, says Suskin. He hopes that the new scheme, and the preliminary work carried out by Schindel, will give an "extra push" to mobility. "We encourage all heads of centres in the United States and Europe to get in touch on their own, and then come to the NSF for some funding," Suskin says.

The use of established centre infrastructures, and the mentorship that they can provide, is essential to counter young scientists' concerns about possible career problems when re-entering the system, says Philip Bucksbaum, an associate director of the Center for Ultrafast Optical Science at the University of Michigan in Ann Arbor.

Funded by an NSF pilot programme, Bucksbaum's centre has, for the past five years, exchanged postdocs with the French Ecole Polytechnique's Laboratory of Optical Applications. The arrangement has included annual bilateral miniconferences between the centres.

"It has worked very well — there were no problems at all with research careers," says Bucksbaum. He calls on young researchers to make use of the opportunities for mobility that such partnerships can present. In career development, "the plusses of a stay abroad will always outweigh the minuses", he says. ■

www.nsf.gov/home/int/europe

France hails milestone in start-ups

Sally Goodman, Paris

Entrepreneurship is finally making a mark on French public research, according to at least one important measure.

Last week the CNRS, France's main basic research agency, celebrated the creation of the 100th start-up company by its researchers — three years after a law was passed allowing such activity for the first time.

"The 1999 law is really behind the change of mentality that we are seeing in French researchers," says Nicolas Mouz, a structural biologist who helped set up Protein'eXpert, a biotechnology company, while on sabbatical from a CNRS laboratory in Grenoble.

The law, which was championed by the research minister at the time, Claude Allègre, opened the door for researchers working in public universities and research agencies to run or own companies built on their research findings. France was later than most of its competitors in allowing publicly funded researchers to do this, and the law's supporters thought that this was constraining innovation in biotechnology and other sectors. The French law also provided around 80 million euros (US\$80

million) to help support such companies.

Geneviève Berger, director general of the CNRS, says that the fostering of these small companies remains a top priority for the agency. It now aims to spin off at least 50 more companies each year from its laboratories, most of which are located in universities. The entrepreneurial culture needs to extend even further into the laboratories, she says, until researchers "are prepared to accept failures".

But Tristan Rousselle, Protein'eXpert's chief executive, says that more needs to be done to pick up on innovations with potential, and to overcome reservations about commercial activity that, he says, still exist at some CNRS laboratories. "The system still relies on resourceful researchers finding ways to promote their own projects," he says.

Researchers can expect France's new centre-right government to push hard for more business start-ups. Research minister Claudie Haigneré says she intends to reinforce her ministry's support for new companies, and the government is also expected to implement tax breaks for businesses that do research. ■



Taking the plunge: a borehole being drilled in California is a pilot for a more ambitious scheme.

EarthScope drillers seek hole truth about San Andreas fault

San Diego In the next few weeks, an international team will finishing drilling a 2.2-kilometre borehole in a seismically active zone of California's San Andreas fault.

The \$2.3-million hole at Parkfield, in the coastal mountains south of San Francisco, is a pilot for a more elaborate project called the San Andreas Fault Observatory at Depth (SAFOD). This aims to investigate the physical and chemical processes that affect the fault zone by sinking a hole to a depth of 4 km. Scientists will extract fluid and rock samples and lower instruments to find out more about the region's seismic activity.

Similar studies will be conducted as part of the test drilling, which is being carried out under the auspices of the International Continental Scientific Drilling Program, based in Potsdam, Germany. "The pilot hole is really a warm-up exercise for SAFOD," says geophysicist Mark Zoback of Stanford University in California. "It was conceived about a year ago as a way to begin studying the upper crust adjacent to the fault zone."

If SAFOD is approved by the US Congress, the first instalment of its \$30-million, six-year funding could be made available next year. It is part of a wider programme called EarthScope, a series of large Earth-science projects backed by the National Science Foundation, the US Geological Survey and NASA (see *Nature* **405**, 390–392; 2000).

♦ <http://icdp.gfz-potsdam.de>
♦ www.earthscope.org

Genentech to pay hospital \$500 million 'royalties owed'

San Francisco Genentech, the world's second-largest biotechnology company, has been hit with a US\$500-million penalty for failing to pay royalties to a Californian hospital whose researchers developed an important genetic-engineering technique.

A jury in Los Angeles set punitive damages at \$200 million on 24 June, two weeks after awarding an initial \$300 million to the City of Hope National Medical Center in Duarte.

Genentech, based in South San Francisco, funded research at City of Hope in the 1970s in exchange for a licence to use the resulting technology, which involved inserting genes for human proteins, such as insulin, into bacteria. The suit alleged that Genentech has sublicensed the technology to two dozen other companies without paying royalties.

This was the second jury to hear the case. The first failed to reach a verdict, voting by seven to five in favour of Genentech last October. Genentech, which recorded \$1.7-billion drug sales last year, says it will appeal.

Pill pioneer leaves Cambridge £45 million

London A multimillionaire chemist who helped to develop the contraceptive pill has left the University of Cambridge £45 million (US\$69 million) — the largest donation it has received from one person. The bequest from Herchel Smith, who died in December, will fund new professorships in physics, pure mathematics, biochemistry and molecular biology, as well supporting exchange visits between Cambridge and Harvard University. Harvard announced earlier this year that Smith had left it some \$100 million.

Born in Plymouth, Smith received a postgraduate degree in organic chemistry from Cambridge before moving to the University of Manchester, where he devised and patented new ways of synthesizing steroids used in the contraceptive pill. He moved to the United States in 1961, where he worked with Wyeth Pharmaceuticals in Philadelphia. He also made donations to both Cambridge and Harvard during his life.

Online protein project aids bioinformatics expansion

San Diego A web-based bioinformatics system that allows researchers to view and analyse microscopic images of proteins at work went online this month.

Called BioSig, the system was created by computer scientists at the Lawrence Berkeley National Laboratory and the National Energy Research Scientific Computing Center. The system is designed to let researchers at multiple locations study protein production, cell morphology and

cellular organization in response to various experimental treatments. It includes algorithms for the automated analysis of digitized microscope images.

It is one of several projects being developed to aid online studies of biological images. Researchers at the University of Oxford, for instance, are working on the BioImage Database, which aims to provide access to a wide range of biological images, and the tools to analyse them.

♦ <http://vision.lbl.gov/Projects/bioinformatic/index.htm>

♦ www.bioimage.org

Study of pet cancers could protect owners

Washington An innovative registry aims to document the incidence of cancer in pet animals. The scientists behind the project hope to tap a useful source of data on the role of environmental contaminants in triggering cancer, free from confounding factors such as alcohol consumption and smoking.

Headed by Rodney Page, director of the Comparative Cancer Program at Cornell University in Ithaca, New York, the Companion Animal Tumor Registry will merge information volunteered by local veterinary surgeons with geographical information and parallel databases of human cancer incidence. Initially, it will focus on two counties in Long Island where there seems to be a high incidence of human breast cancer, although it may later be extended to other areas of New York State and beyond.

Although pet animals live in the same environment as humans, they are often more exposed to potential carcinogens such as the chemicals sprayed on lawns, and so may serve as sentinels for human cancer risks.

♦ www.cfe.cornell.edu/bcerf/anireg.cfm

Use of 'anti-HIV' spermicide may increase infection risk

Paris A commonly used spermicide, nonoxynol-9, once thought to offer protection against HIV infection, may

actually increase the risk of contracting the virus, according to the World Health Organization (WHO).

Although it is primarily used as a contraceptive, laboratory tests in the 1980s showed that nonoxynol-9 could also inactivate HIV. But according to a report issued by the WHO last week, recent clinical trials show that, when used frequently, nonoxynol-9 can increase the risk of infection, probably by disrupting the epithelial lining of the vagina or rectum.

An effective vaginal microbicide would offer an important form of protection against HIV infection in many developing countries, where it is culturally difficult for women to insist on the use of condoms. The New York-based Rockefeller Foundation, which is leading an initiative to increase research into microbicides, estimated earlier this year that a product could be brought to the market by 2007, if the work was backed by further funding and the necessary political will. A number of potential candidates are close to entering clinical trials, including a gel derived from seaweed.

Tiny cracks ground NASA's shuttle fleet

Washington With NASA's space-shuttle fleet mostly devoted to building the International Space Station nowadays, research missions have become a rarity. Scientists eager to conduct microgravity experiments on the shuttle will now have to wait a while longer, after NASA grounded the entire fleet because of a mechanical flaw.

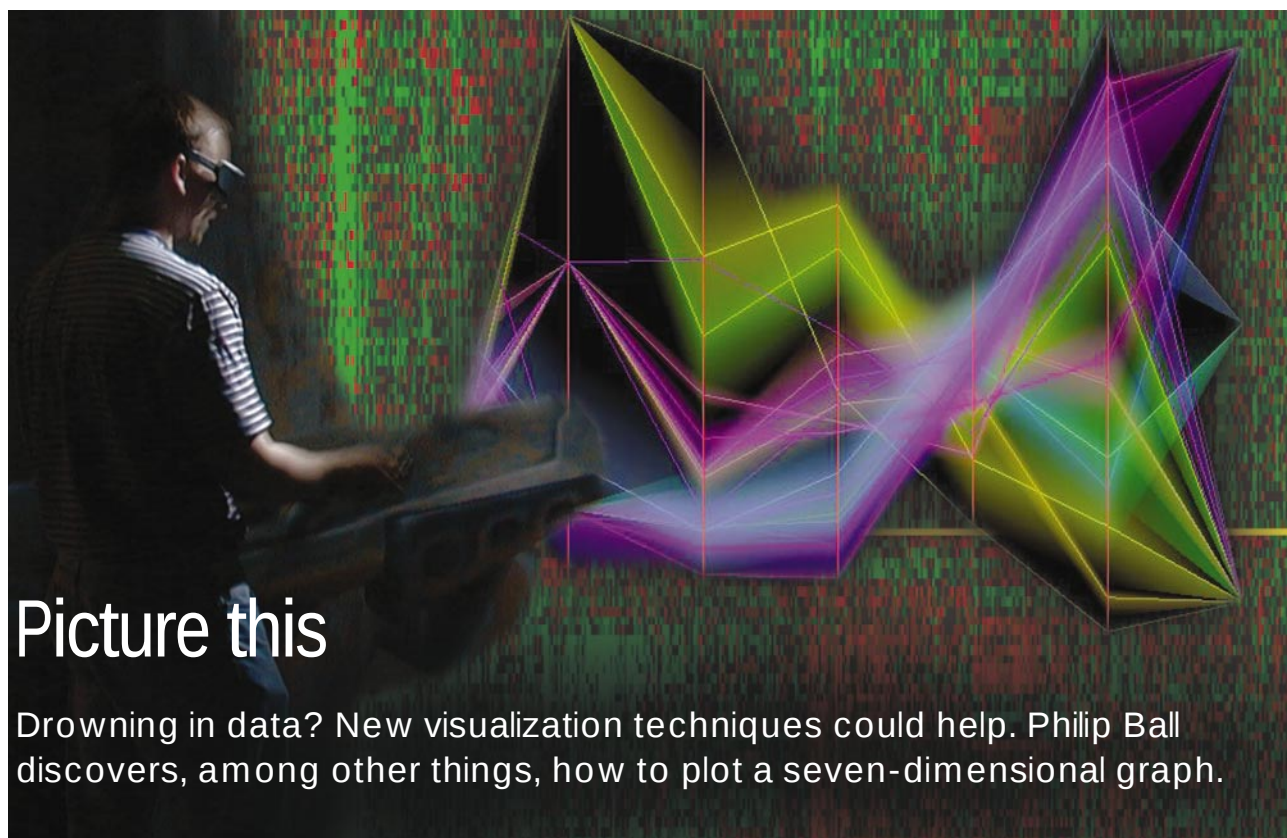
The STS-107 mission was scheduled for launch on 11 July, but will now be delayed a month or more while NASA investigates tiny cracks in the linings of propellant lines in the shuttle *Columbia*'s main engines. Engineers are now assessing the seriousness of the damage and how long it might take to repair.

A dedicated research mission, STS-107 includes more than 80 experiments in fields ranging from combustion, through protein-crystal growth, to astronaut health.

♦ <http://spaceresearch.nasa.gov/sts-107>



Sitting it out: STS-107's crew, seen in training last year, are waiting for their launch to be rescheduled.



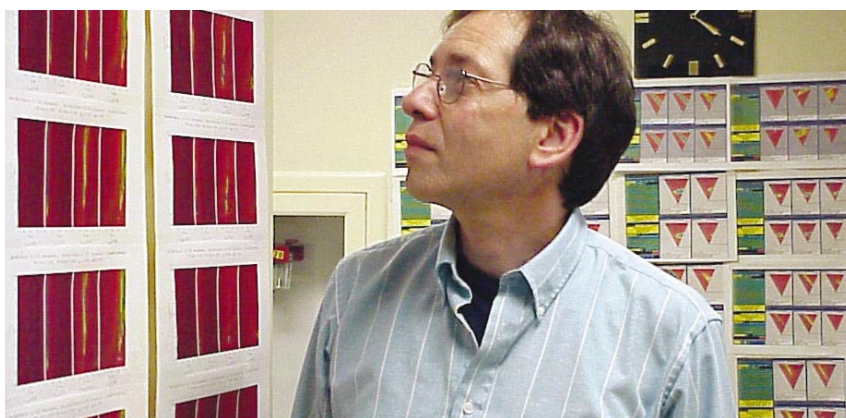
Picture this

Drowning in data? New visualization techniques could help. Philip Ball discovers, among other things, how to plot a seven-dimensional graph.

Ralph Kahn's office at NASA's Jet Propulsion Laboratory in Pasadena, California, has a unique line in interior decor. Brightly coloured charts cover the walls. Red is a favourite theme, streaked through with yellow and blue. The garish creations won't win any design awards, but Kahn hopes that by staring at them for long enough, he could come up with new ideas about the Earth's climate.

Kahn has created his unusual wallpaper because, like many other scientists, he is swamped with data. The figures illustrate measurements taken by a sensor on board the Terra satellite, the flagship of NASA's Earth-observation programme. The device produces data at a phenomenal rate — its nine cameras simultaneously monitor four frequencies of sunlight reflected by the Earth. "To understand a climate phenomenon involving multiple feedbacks, you really need to look almost everywhere, in detail, and often," explains Kahn.

By viewing plots of the Terra data, Kahn hopes to spot hints of patterns that are worthy of analysis. Yet the possibilities for plotting data in revealing ways run far beyond Kahn's two-dimensional coloured graphs. Few people are aware of it, but sophisticated methods for visualizing large data sets are within the reach of most researchers. Thanks to increases in computing power and improvements in graphical software, scientists can now make more



Paper chase: Ralph Kahn hopes to spot patterns in his data by literally surrounding himself with it.

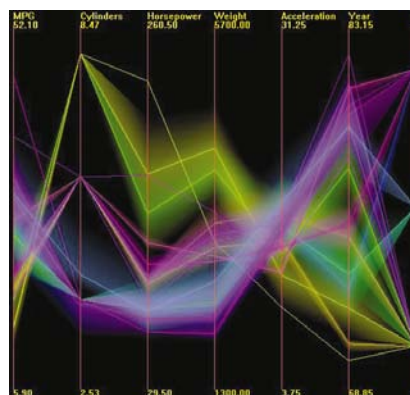
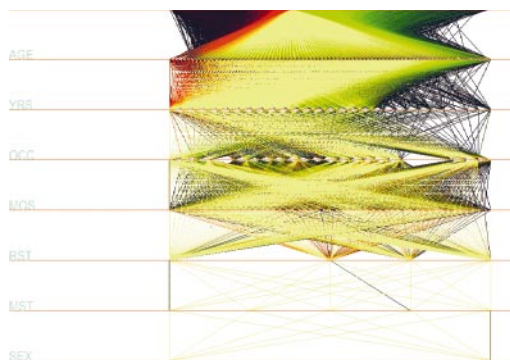
and more use of the best pattern detector that we have at our disposal — the human visual system.

Digital deluge

Researchers studying everything from gene expression to financial risk assessment stand to benefit. Many disciplines routinely produce more data than researchers know what to do with. In 1999, for example, an advisory committee to the US National Institutes of Health estimated that some biomedical laboratories can produce up to 100 terabytes (10^{14} bytes) of information a year — enough to fill a million encyclopaedias. Digital technology

is the main culprit. "A $2,048 \times 2,048$ -pixel colour image contains 16 megabytes of data, even if it's a picture of my kitchen floor," points out Bill Eddy, a statistician at Carnegie Mellon University in Pittsburgh, Pennsylvania.

It's not just a question of having too much data — the kind of data also matters. The relationships between two variables can be examined using a simple graph. But experiments in many fields generate data using different instruments that simultaneously measure several parameters, which may or may not be related. The number of variables that could be paired in a two-dimensional plot is enormous — a problem known as



Read between the lines: parallel coordinates can show which factors put bank customers at risk of slipping into the red (left), and the variables that are linked to efficient performance in cars (right).

the 'curse of dimensionality'.

It is here that visual data-mining could help. Before pinning down precise mathematical relationships within a data set, visual techniques provide a first line of attack that can suggest what kind of trends and patterns may lie within the numbers, and may thus help guide the focus of more detailed analysis. Such an approach, known as exploratory data analysis, has long been used by statisticians, but sophisticated visualization techniques are now playing an increasingly important part in it.

DNA microarray experiments, which can simultaneously monitor the expression of thousands of genes, are already benefiting from visual analysis. David Botstein, a geneticist at Stanford University who helped to pioneer microarray studies, began working on the problem in 1998. Together with colleagues, he described how data on the activity of thousands of genes can be displayed in a way that makes it clear which genes have similar patterns of expression¹.

In January, a team from the Netherlands Cancer Institute in Plesmanlaan and Rosetta Inpharmatics, a drug-development and technology company in Kirkland, Washington, used a similar plotting method in their work

on predicting the course of breast cancers². The team looked at tissue samples from the tumours of almost 100 patients, some of whom had gone on to develop secondary cancers in the five years after the samples were taken.

The researchers measured expression levels for almost 5,000 genes in each tumour, and used a mathematical algorithm to create a list of tumours in which genes with similar patterns of expression were grouped together. Plotting the list using colours to represent expression levels revealed that the tumours seemed to fall into two different groups (divided by the yellow line in the plot; see below left). Further investigation helped the team to identify a subset of 70 genes that could be used to predict whether a patient will develop secondary tumours.

Botstein feels that other important findings lie hidden in microarray data. "There is more information in the data than we as a community have yet extracted," he says. "I'm hopeful that better things may emerge. This is the very beginning."

Working in parallel

Other visualization techniques have longer histories, but are now benefiting from improvements in computing technology. One example, the method of parallel coordinates, was first proposed during the 1980s, and is particularly useful for tackling high-dimensional data.

In the demonstration example shown in the plot above (right), the method is used to represent performance data for cars. Each vehicle is described by seven variables, such as weight and acceleration. Parallel axes are used to represent each variable, and the data for each vehicle form a line that links the values on each axis. Trends can then be extracted by looking for sets of lines that cluster together. In the example given here, representing cars with high fuel efficiency in purple shows that these vehicles tend to have low weights and to be more recent models.

More complex data can be mined by combining parallel coordinates with other

visualization techniques. Edward Wegman, a statistician at George Mason University in Fairfax, Virginia, has used these methods to identify correlations in data on bank customers in order to find out whether particular combinations of variables can be used to identify individuals who are at risk of financial problems³.

Each customer is classified by eight variables, such as age, occupation and profit status with the bank. Plotting all of the records — over 130,000 in total — produces a solid mass of lines that is difficult to interpret, so Wegman used a technique known as saturation brushing to reveal hidden patterns. Each line was given a hint of colour: red for those with overdrafts, green for those in credit. Individual lines that were brushed in this way still appeared black, but the colours added up when lines overlapped. If, for example, a group of overdrawn customers clustered around a point on one of the axes, the mass of lines appeared red. Roughly even numbers of red and green lines mixed around points that did not correlate with credit status, and these points appeared yellow.

The colour of money

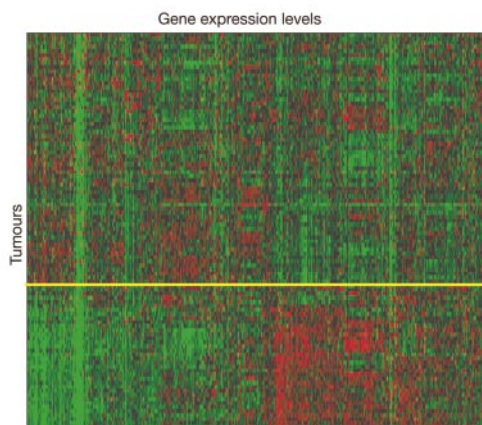
Colour brushing revealed, unsurprisingly, that younger customers are more likely to be in debt (see plot, above left). But Wegman suspected that more interesting correlations might be hidden in the data, and so he used a further method of analysis, known as a grand tour. This amounts to taking a virtual journey through the multi-dimensional data-space so that the points — or lines in parallel coordinates — are seen from many different perspectives.

The process is easy to visualize in three dimensions. Imagine a cloud of points, shaped like a doughnut, on a three-dimensional graph. If the graph is viewed through the plane of the doughnut, the hole is hidden. It is only when the data are seen from other angles that the overall structure becomes clear.

A mathematical version of this process can be applied to data of any number of dimensions. In the case of parallel coordinates, the lines are viewed from a new position by replotting the data using axes that represent combinations of the original variables, rather than assigning a single variable to each axis.

As the grand tour of the bank data progressed, interesting features emerged. Wegman clarified these features by removing nearby points that did not appear to be part of potential trends, a process known as data-pruning. After several successive plots, the data revealed further risks associated with certain occupations, whether the customer owns or rents their home, and their marital status.

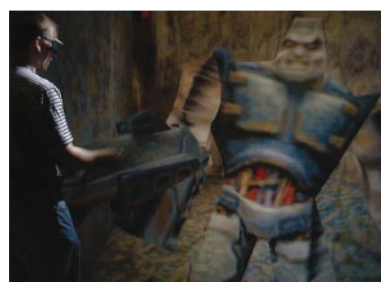
Because each axis now represents a combination of variables, risks cannot easily



Colour-coded: this plot of gene-expression data shows breast tumours falling into two groups.



Business or pleasure? Paul Rajlich uses the CAVE to interact with data and, in his spare time, to tackle some virtual baddies (inset).



be related to the original variables, such as occupation and so on. But the risk associated with a new customer can still be calculated by combining that person's data in the appropriate way. And by not singling out particular factors, the bank can avoid accusations of discrimination.

Other visualization researchers stress the importance of interacting with data. Jim Thomas, an expert in data-mining technology at Pacific Northwest National Laboratory in Richland, Washington, believes that the key to success lies in the dynamic aspect of modern computer graphics. "Interaction is as important as the visuals," he says. And if users want to get really interactive, they can no better than to enter a device known as the CAVE.

Developed in the early 1990s in the Electronic Visualization Laboratory (EVL) at the University of Illinois at Chicago, the CAVE is a cube-shaped room, around three metres square, with one open wall through which three-dimensional computer images are projected onto the other walls and floor.

Inside the CAVE, users can 'float' in data-space. Sensors track head movements, and the on-screen graphics are adjusted so that the image changes just as it would if the user were moving around a real scene. A manual controller called the wand acts like a kind of three-dimensional mouse, allowing users to probe and modify the images.

"The CAVE allows you to understand very complicated structures because you can intuitively and easily manipulate your view," says Paul Rajlich, a CAVE researcher at the National Center for Supercomputing Applications (NCSA) at the University of Illinois at Urbana-Champaign. "You can simply walk around the CAVE and examine the structure just as if it were a real object."

There are now about a hundred CAVEs in use around the world. Most are used in virtual-reality research, automotive design and medical training. But Earth scientists often use them to visualize geological structures, and biologists are also getting in on the act. Timothy Karr studies fertilization in fruitflies at the University of Chicago. He has used a software system known as Crumbs, developed at the NCSA and other departments at Urbana-Champaign, which allows users to explore paths through three-dimensional data-space by dropping 'crumbs' to mark their trails.

Gathering the crumbs

Karr is interested in the fate of fruitfly sperm after it enters the egg. He used a CAVE at the NCSA to display microscopic images of sperm tails inside the egg. By using Crumbs to outline the tail on successive images, he was able to plot how the sperm changes once it is within the egg⁴. "Think about trying to follow an unravelling ball of yarn inside an egg, and you have an idea of how difficult this would be without software like Crumbs," says Karr.

CAVEs, and other less sophisticated methods, could help researchers get a handle on the vast, high-dimensional data sets that are becoming increasingly common. But the techniques are not without their problems. Designing visualization methods is a multi-disciplinary task, drawing in statisticians, computer programmers and the researchers who produce the data, yet these different groups do not always communicate effectively. And even when techniques are well-designed, some researchers are wary of placing too much confidence in them.

Many of the potential problems stem from a lack of cooperation, an issue that Botstein has experienced in his microarray

work. "Biologists know too little about mathematics and computation, and computer scientists and statisticians know too little biology," he says. "And as in any interdisciplinary endeavour, there are cultural and value differences that cause misunderstanding." Biologists, says Botstein, do not particularly care how they analyse their data, as long as it offers some insight. But mathematicians may feel that a 'trivial' analysis has no academic value, despite the fact that such methods often stretch biologists' analytical powers to the limit.

There is also a divide between researchers who work on scientific images and those that use visualization as a precursor to statistical analysis. "The two groups tend to have their own separate conferences," says Jason Leigh of the EVL. "There are so many techniques from both camps that are mutually beneficial to one another, but the opportunities for cross-fertilization are lost."

Others lament the fact that vision researchers are seldom involved in developing the techniques, and warn that statisticians could be placing too much confidence in the pattern-recognition abilities of the human brain. "The human visual system is remarkably good at discovering real patterns," says Eddy. "But it is also remarkably good at discovering false patterns. The experts who work on visualization methods are, with a few notable exceptions, woefully ignorant of the relevant psychology and psychophysics. Sadly, the cognitive psychologists have bigger fish to fry and are not especially interested."

If the groups do start working together, they are likely to find that the speed with which data become redundant creates a demand for new methods of analysis. "The time window for analysis is getting shorter and shorter," says Daniel Carr, a statistician at George Mason University. "It is hard to imagine that in five years scientists will be much interested in analysing today's microarray data."

They will also find that with data piling up in many different disciplines, and becoming less useful increasingly quickly, there has never been a greater need for visualization methods. And for the advocates of visualization, the benefits are clear. "Science will continue to progress with or without more attention to visualization and data analysis," says Eddy. "But it will progress much faster with careful attention to these issues. To begin with there has to be a will to do it — a commitment to the analysis." ■

Philip Ball is a consultant editor of *Nature*.

1. Eisen, M. B., Spellman, P. T., Brown, P. O. & Botstein, D. *Proc. Natl. Acad. Sci. USA* **95**, 14863–14868 (1998).
2. van 't Veer, L. J. et al. *Nature* **415**, 530–536 (2002).
3. Wegman, E. *Stat. Med.* (in the press).
4. Karr, T. L. & Brady, R. *Trends Genet.* **16**, 231–232 (2000).

Your destiny, from day one

The mammalian body plan starts being laid down from the moment of conception, it has emerged. Helen Pearson considers the implications of a surprising shift in embryological thinking.

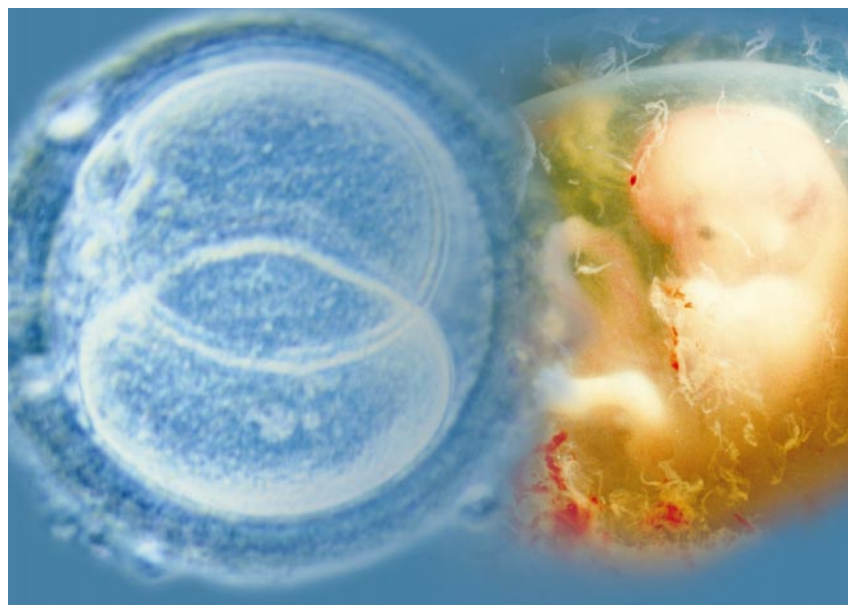
Your world was shaped in the first 24 hours after conception. Where your head and feet would sprout, and which side would form your back and which your belly, were being defined in the minutes and hours after sperm and egg united.

Just five years ago, this statement would have been heresy. Mammalian embryos were thought to spend their first few days as a featureless orb of cells. Only later, at about the time of implantation into the wall of the uterus, were cells thought to acquire distinct 'fates' determining their positions in the future body.

But by tagging specific points on mammalian eggs shortly after fertilization, researchers have now shown that they come to lie at predictable points in the embryo. Rather than being a naive sphere, it seems that a newly fertilized egg has a defined top–bottom axis that sets up the equivalent axis in the future embryo. Controversially, one group even claims that the spot on the egg at which the sperm enters determines where the first cell division occurs — and that the resulting two cells already have a bias towards different fates.

This new understanding opens fresh avenues of study for developmental biologists. But it also raises the possibility that any technique that meddles with early human development — such as the removal of cells from an early embryo for pre-implantation genetic testing — might potentially be harmful. "It's possible you could be removing a cell with a predictable fate and causing damage," says Alan Handyside, who studies embryo abnormalities at the University of Leeds, UK.

Biologists have long known that the eventual axes of the embryo in most species are laid down either before fertilization, or in the first hours afterwards. In fruit flies, for



Axes established in the two-celled embryo (left in this montage) set up those in the fetus.

instance, the egg inherits a molecule that is more concentrated at one end of the egg than the other, and thus defines the head–tail axis.

Heads or tails?

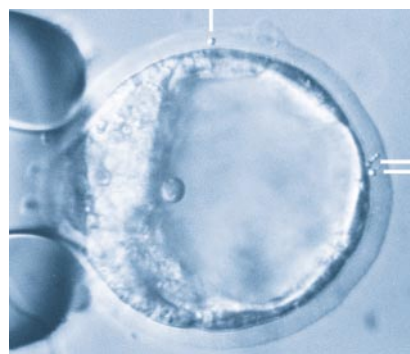
But mammalian embryos were considered to be a special case. First, they have a striking ability to compensate for damage. Split up the first two cells of a mouse embryo and both recover to make two apparently normal mice. Second, only around 15% of cells in the blastocyst — a hollow sphere of cells that forms some five days after conception — contribute towards the body proper, rather than supporting tissues such as the placenta. These cells reside in a structure called the inner cell mass (ICM). Finally, the first visible sign of a distinguishable head or tail takes 6.5 days to appear in mouse embryos. "All that argued against the idea of there being a map on the egg," says developmental biologist John Gurdon of the Wellcome/Cancer Research UK Institute of Cancer and

Developmental Biology in Cambridge.

The first hint that the blastocyst was not the unassuming orb it appeared came in the 1980s. Two little-noticed studies from Jean Smith of Queen's College in Flushing, New York, showed that the mouse blastocyst, rather than being a symmetrical sphere, is slightly distorted and has recognizable axes^{1,2}. What's more, these axes appeared to match up with those of the fetus, suggesting that the former sets up the latter.

The findings prompted Richard Gardner, an embryologist at the University of Oxford, UK, to repeat the work, drawing similar conclusions³. But it took another five years before Gardner could make

This way up: Richard Gardner injected oil drops into two-celled embryos (below), giving markers that showed up in the blastocyst (below right).



anyone listen. "People were quite hostile," he recalls.

Gardner suspected that the axes present in the blastocyst were there from the moment of conception. But to show that a specific point on the fertilized egg consistently maps to a particular position on the embryo, he needed a way of tagging the egg without disturbing it. He found such a marker in the form of the second polar body, a 'spare' set of chromosomes thrown out of the egg when the sperm enters: it remains glued to the embryo's surface in a set position.

Impossible to ignore

Examining blastocysts, Gardner found that the polar body consistently perched on a line of latitude dividing the upper hemisphere, containing the ICM, from the lower hemisphere⁴. This suggested that the top and bottom of the egg line up with, and may determine, the left and right sides of the blastocyst. He backed up this idea by using oil drops placed in the jelly-like protein coat of two-cell embryos to trace cell axes more accurately⁵. "People could no longer ignore that there was patterning information in the egg," says Gardner.

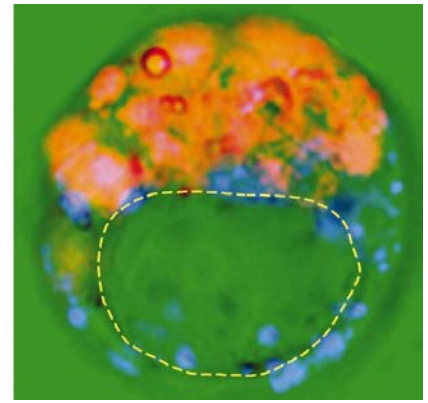
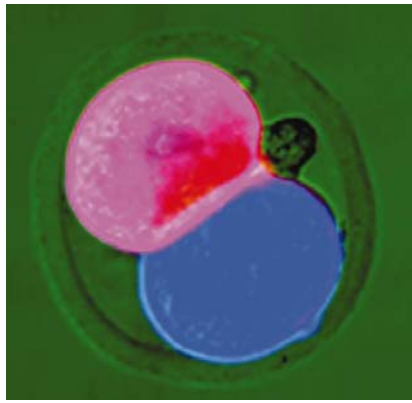
Meanwhile, Magdalena Zernicka-Goetz's team at the Wellcome/Cancer Research UK Institute had found that this pattern is retained by the embryo after implantation. The researchers took unimplanted blastocysts and labelled cells at one or other pole of each with a fluorescent protein before transferring them into female mice and allowing them to implant. After 6.5 days, these cells ended up towards either one end or the other of the embryo⁶.

But how does the initial pattern get there? Zernicka-Goetz suspected the act of fertilization itself was the key, and injected sticky fluorescent beads under the coat of mouse eggs at the spot where sperm had penetrated. In most cases, the bead's position roughly coincided with the equator of the first cell division, implying that the sperm's entry point determines where the cell first divides⁷.

In subsequent experiments, Zernicka-Goetz painted the first two cells, one red, one blue, using dyes dissolved in olive oil. She then tracked their descendants into the blastocyst. One cell usually gave rise to the region containing the ICM, the other to the region largely destined to make the placenta and other supporting tissues⁸.

Zernicka-Goetz's conclusion is that the first division of the egg influences the fate of each cell and ultimately, all the tissues of the body. "There is a memory of the first cleavage in our life," says Zernicka-Goetz.

Gardner disputes the idea that the sperm entry point is critical, arguing that Zernicka-Goetz's fluorescent beads are drifting away from the point of fertilization. In recent work, he used components of the sperm's discarded tail to mark its entry position into



Dyeing to know: Magdalena Zernicka-Goetz used different-coloured stains to track the descendants of the first two embryonic cells (above left).

the egg, and found no association with the equator of the first cell division⁹. Zernicka-Goetz has countered with a third way of marking the entry site using fluorescently labelled sperm that transfer their label to the egg — and re-asserted her original conclusion¹⁰. She is now working on eggs triggered into developing without sperm. If the first cell division in these embryos produces cells that contribute more equally to each half of the blastocyst, it will boost her theory that the point of sperm entry is the key factor.

Patterns pending

Developmental biologists are now keen to work out the molecular mechanisms underlying the patterning information in early mammalian embryos. As in fruit flies, they may contain an asymmetrically distributed 'determinant', a molecule that influences cell fate and is inherited unequally in the first cell division. Jonathan Van Blerkom of the University of Colorado at Boulder has intriguing evidence that two proteins are distributed in this way in human and mouse eggs¹¹. He does not believe that these molecules are the determinants, but rather that their distribution is determined by the action of a yet-to-be-discovered mechanism.

Other researchers suspect that the sperm's entry on one side triggers a complete reorganization of the egg's internal skeleton that then makes cells at different positions in the embryo divide at slightly different times.

Another mystery is how early mammalian embryos retain the ability to develop normally if damaged or split in two, given the existence of patterning information that appears

to narrow down cell fate. Most researchers think that the patterning information is quite weak, so that cells become biased towards producing certain tissues, rather than irrevocably committed. Only later are the biases stabilized and cell fates fixed.

Nevertheless, the existence of patterning information in the early human embryo raises the issue of whether certain assisted-reproduction techniques could disrupt the delicate processes of establishing body axes.

If sperm entry point is an important factor, for instance, that throws up questions about intra-cytoplasmic sperm injection, in which sperm from infertile men are injected directly into the egg. Pre-implantation genetic testing, in which two cells are removed from an eight-cell embryo to test for inherited diseases such as cystic fibrosis, is another area of concern. "Perhaps we should pay attention to which cells we remove," says Handyside. But other experts believe that the flexibility of human embryos is sufficient to compensate for these manipulations. Damaged embryos may, in any case, spontaneously abort.

What is clear is that developmental biologists will no longer dismiss early mammalian embryos as featureless bundles of cells — and that leaves them with some work to do. "I believe in the new philosophy," says Tom Fleming, a developmental biologist at the University of Southampton, UK, "but there's a lot of detail yet to be understood."

Helen Pearson works in Nature's news syndication team.

1. Smith, L. J. *J. Embryol. Exp. Morph.* **55**, 257–277 (1980).
2. Smith, L. J. *J. Embryol. Exp. Morph.* **89**, 15–35 (1985).
3. Gardner, R. L., Meredith, M. R. & Altman, D. G. *J. Exp. Zool.* **264**, 437–443 (1992).
4. Gardner, R. L. *Development* **124**, 289–301 (1997).
5. Gardner, R. L. *Development* **128**, 839–847 (2001).
6. Weber, R. J., Pedersen, R. A., Wianny, F., Evans, M. J. & Zernicka-Goetz, M. *Development* **126**, 5591–5598 (1999).
7. Piotrowska, K. & Zernicka-Goetz, M. *Nature* **409**, 517–521 (2001).
8. Piotrowska, K. et al. *Development* **128**, 3739–3748 (2001).
9. Davies, T. J. & Gardner, R. L. *Hum. Reprod.* (in the press).
10. Plusa, P., Piotrowska, K. & Zernicka-Goetz, M. *Genesis* **32**, 193–198 (2002).
11. Antcsak, M. & Van Blerkom, J. *Mol. Hum. Reprod.* **3**, 1067–1086 (1997).

Population should be on the Johannesburg agenda

High fertility rates continue to threaten both the environment and human well-being.

Sir — On 26 August, the United Nations (UN) World Summit on Sustainable Development in Johannesburg will consider strategies with a far broader mandate for action than the UN Conference on Environment and Development in Rio de Janeiro in 1992. Population as a key component of sustainable development should figure prominently on the Johannesburg agenda. Yet, after four preparatory meetings for Johannesburg, the topic is still absent.

If we do not put the human population at the core of the sustainable-development agenda, our efforts to improve human well-being and preserve the quality of the environment will fail.

The Johannesburg Summit must heed the first principle of the 1992 Rio Declaration — that “human beings are at the centre of concern for sustainable development” — by taking full account of how population and society interact with the natural environment. This is one of the basic conclusions of the Global Science Panel on Population and Environment, an independent body of experts organized by the International Institute for Applied Systems Analysis (IIASA), the International Union for the Scientific Study of Population and the United Nations University.

Sustainable development aims at improving human well-being, particularly through alleviating poverty, increasing gender equity, and improving health, human resources and stewardship of the natural environment. Because demographic factors are closely linked to these goals, strategies that take population into account have a better chance of success.

The International Conference on Population and Development in Cairo in 1994 recognized that population policy should be oriented towards improving social conditions and expanding choices for individuals. The key recognition was that focusing on people — their rights, capabilities and opportunities — would have multiple benefits for individuals, for societies and for their sustainable relationship with the environment. Therefore, in Johannesburg, consideration of sustainable-development policies must include population growth and distribution, mobility, health impacts of environmental change, differential vulnerability, and the empowerment of people, especially of women.

Fertility decline in high-fertility

countries, by slowing population growth, can make many environmental problems easier to solve. It can also have important economic benefits through reducing the number of children relative to the working-age population, creating a unique opportunity to increase investments in health, education, infrastructure and environmental protection.

In high-income countries, the environmental impact of population growth and distribution must be considered jointly with high consumption rates. Even in countries where little growth is envisioned, unsustainable patterns of consumption have global implications for the environment and human well-being, and must be addressed with appropriate policies.

Hence, on the way from Rio to Johannesburg we must go through Cairo. Two key policies are needed: first,

investment in voluntary family planning and reproductive-health programmes; and second, education and empowerment, especially of women, in order to reduce fertility, enhance individual choice, contribute to greater environmental awareness and reduce vulnerability to environmental changes.

Wolfgang Lutz, Mahendra Shah

Coordinators, Global Science Panel on Population and Environment (GSPPE), IIASA, Schlossplatz 1, A-2361 Laxenburg, Austria

Other signatories of this letter are the panel members of the GSPPE:

R. E. Bilsborrow, J. Bongaarts, P. DasGupta, B. Entwistle, G. Fischer, B. Garcia, D. J. Hogan, A. Jernelov, Z. Jiang, R. W. Kates, S. Lall, F. L. MacKellar, P. K. Makinwa-Adebusoye, A. J. McMichael, V. Mishra, N. Myers, N. Nakicenovic, S. Nilsson, B. C. O'Neill, X. Peng, H. B. Presser, N. Sadik, W. C. Sanderson, G. Sen, M. F. Strong, B. Torrey, D. van de Kaa, H. J. A. van Ginkel, B. Yeoh, H. Zurayk.

Full address details and affiliations are available on the panel's website at www.iiasa.ac.at/gsp

Beautiful vistas, but is this really science?

Sir — Negotiating Stephen Wolfram's vast book *A New Kind of Science*, on which you report in the News Feature “What kind of science is this?” (*Nature* **417**, 216–218; 2002), reminded me of Huckleberry Finn's epic journey drifting down the Mississippi on a raft — not just the length, but the scenery.

On the left bank of the river we pass a long series of wondrously complex and often very beautiful pictures, the amazing products of his simple automata. On the right bank we drift by his conjectures and speculations about the meaning of these gorgeous images. He ‘explains’ nearly all the real world's puzzling phenomena, from particle physics to evolution of species.

How might we cross safely from the left bank of the river to the right? I found myself yearning for a solid bridge to link at least one of Wolfram's pictures to the real world, but unfortunately, all he offered was a leap of faith.

Which leads me to wonder: what did we really see on this long and dazzling journey? If it was ‘science’, even of a new kind, it should fulfil at least two criteria. First, it should be falsifiable: it should be possible to design an experiment to disprove a proposed link between the model and the real world. And second, it should generate new hypotheses about the real world which scientists can test in their laboratories. Perhaps Wolfram could enlighten us: how might his computer

models fulfil either of these criteria in order to qualify as science?

Michael Phillips

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Solve postgrad problems to attract new scientists

Sir — We agree with your Opinion article “Selling science to the young” (*Nature* **417**, 1; 2002) that science should highlight those who turn their scientific training into a more lucrative career. Take, for example, Jan Peter Balkenende, who turned his economics professorship into a premiership after his Christian Democrat party won the Dutch elections in May.

However, it should be stressed that, in addition, the best way of countering the anti-science trend is to solve science's main problems. As you state, the young are refusing to choose a scientific education. Surely it is time to acknowledge fully the complaints of postgraduate associations, to discuss these and to offer improved career prospects and financial rewards?

T. L. Raoul Tan*, Simone Lohner†

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Evolution under pressure

A look at the controversy about industrial melanism in the peppered moth.

Of Moths and Men: Intrigue, Tragedy and the Peppered Moth
by Judith Hooper

Fourth Estate: 2002. 397 pp. £15.99

Jerry A. Coyne

A colleague described the physician and naturalist Bernard Kettlewell as “the best naturalist I have ever met, and almost the worst professional scientist I have ever known”. And yet Kettlewell became one of the best-known evolutionary biologists, for his work produced the canonical example of evolution in action: industrial melanism in the peppered moth (*Biston betularia*). In *Of Moths and Men*, the science journalist Judith Hooper produces a lively history of this work, illustrated with fascinating but disturbing portraits of the principals. These include the ambitious but insecure Kettlewell, ill at ease in the rarefied atmosphere of Oxford University, and his mentor E. B. Ford, a foppish, manipulative man who used and misused Kettlewell in his own quest for fame. Hooper contends that the *Biston* story is not only wrong, but probably fraudulent.

The scientific facts are familiar to biologists. The normal form of *B. betularia* (known as *typica*) is white, speckled with dark markings. In around 1850, a melanic, all-black form produced by a dominant mutation (*carbonaria*) became more numerous in England, at the same time as a rise in industrial pollution. By 1900, *carbonaria* reached a frequency of nearly 100% in manufacturing areas such as Birmingham. There was a parallel increase in industrial areas of the United States, although somewhat later. These evolutionary changes were reversed in the middle of the century when antipollution laws took effect, and *typica* once again became predominant both in Britain and in the United States.

The rise of



Bernard Kettlewell was an enthusiastic naturalist who loved to surround himself with his work.

melanism offered Ford and Kettlewell an unparalleled chance to document and understand natural selection in the wild. Ford suggested, on the basis of breeding experiments, that *carbonaria* was more resistant than *typica* to pollution, and hence survived better. As a naturalist, Kettlewell believed that selection might be due to sharp-eyed birds that preyed on the moths that were most conspicuous in their local habitat.

Kettlewell tested his hypothesis in 1953. After releasing marked *typica* and *carbonaria* in a wood where trees were darkened by pollution, he recaptured a much higher percentage of dark than light moths, implying selective predation on *typica*. Two years later, he obtained the opposite result in an unpolluted forest. Combined with observations of the hunting behaviour of birds, and of the distribution of the two forms in Britain, these experiments seemed to provide Darwin's ‘missing evidence’: a complete story connecting ecological forces to evolutionary change. Kettlewell gained instant fame, and the *Biston* story was quickly installed in biology textbooks, where it remains to this day.

Hooper suggests, however, that the release experiments were probably fudged to achieve the desired outcome. Unfortunately, in her desire to write a lepidopteran ‘whodunnit’, she advances a flimsy

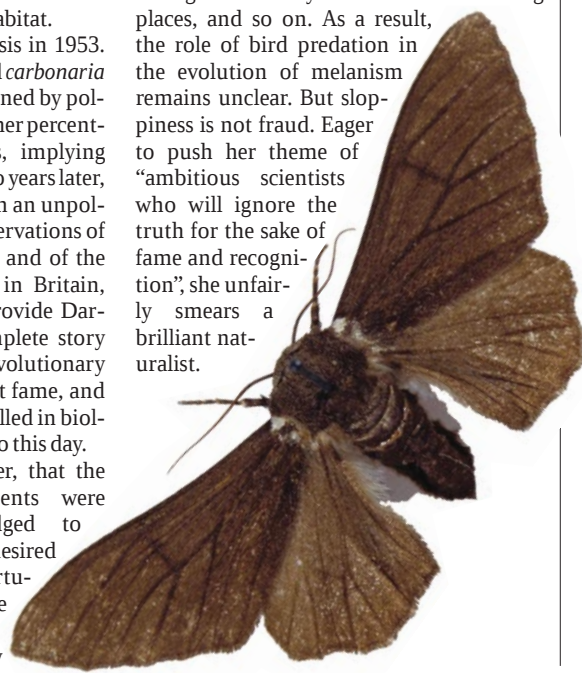
conspiracy theory. Her evidence rests largely on the fact that Kettlewell's first experiment began as a failure, for he recaptured too few moths to show differential survival. His forlorn letter to Oxford provoked a soothing response from Ford: “It is disappointing that the recoveries are not better... However, I do not doubt that the results will be very worth while.”

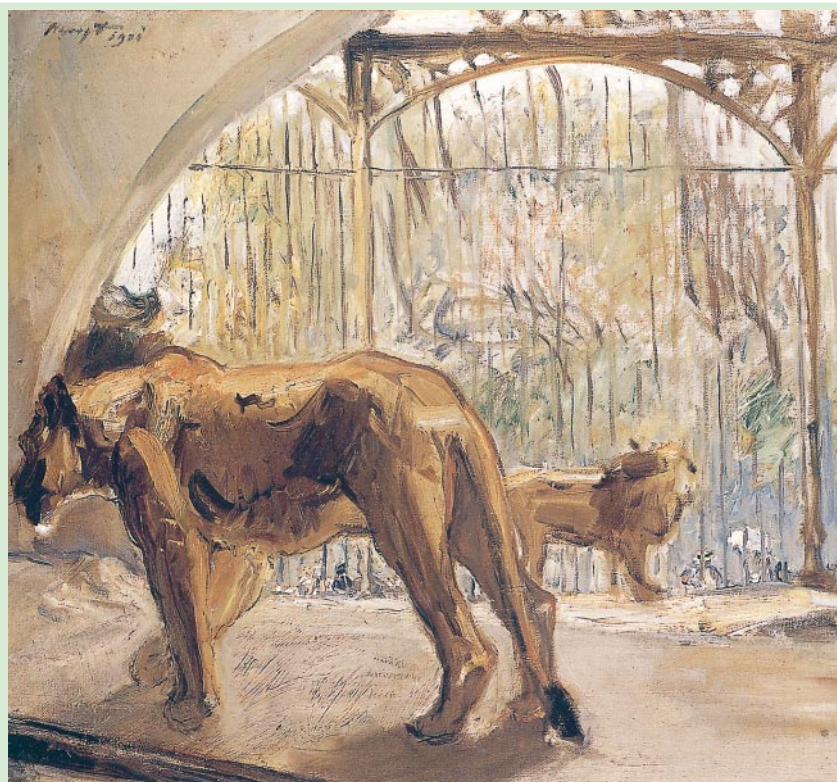
To a professional biologist this sounds familiar; many of us have offered similar consolation to students having a hard time in the field. But to Hooper, Ford's note is a coded message telling Kettlewell to get the right results at all costs. Sure enough, Kettlewell's recapture rate shot up, perhaps because he tripled the number of moths released. Hooper, however, finds this cause insufficient. Using meteorological records, she rules out a change in the weather, and suggests that the increased recapture rate reflected chicanery by Kettlewell — perhaps he changed the experimental design in mid-stream, or even mis-scored the moths. But many factors other than the weather can change recapture rates, including experimental modifications, such as relocating moth traps, that are completely innocuous. Anyone with experience of fieldwork knows how unpredictable such experiments can be.

It has been widely recognized that Kettlewell's experiments were indeed flawed. Hooper enumerates the familiar problems: Kettlewell used mixtures of wild-caught and lab-reared moths, released them at the wrong time of day onto unnatural resting places, and so on. As a result, the role of bird predation in the evolution of melanism remains unclear. But sloppiness is not fraud. Eager to push her theme of “ambitious scientists who will ignore the truth for the sake of fame and recognition”, she unfairly smears a brilliant naturalist.



Light work? The two forms of the peppered moth make it ideal for studies of evolution.





Kept in captivity

Lionesses Pacing in a Cage by Max Slovegt depicts the experience of wild animals put on public display. From *Zoo: A History of Zoological*

Gardens in the West by Eric Baratay and Elisabeth Hardouin-Fugier (Reaktion Books, £28, \$40), recently translated from French.

Many of the problems with Kettlewell's experiments and the 'classic' *Biston* story were first aired by the US biologist Ted Sargent. Curiously, when turning from Kettlewell to Sargent, Hooper's criticality evaporates. She claims that Sargent's criticisms of the moth work ruined his career by making him a pariah, rejected by a scientific establishment enamoured with *Biston*. But this is hyperbole. Sargent's career may have languished because he often published in little-known journals or (as Hooper notes) refused to apply for grants — the kiss of death for a US scientist.

Hooper also champions Sargent's view that industrial melanism was a case not of evolution but of "phenotypic induction" — a developmental change in the colour of moths, presumably caused by the larval ingestion of pollutants. But she conveniently glosses over the simple and unassailable fact that the light and dark alleles of *Biston* segregate as mendelian variants when tested under uniform experimental conditions. Perhaps Hooper embraces the induction theory because it makes for a better story, but surely good science journalism demands that drama takes a back seat to data. Numerous scientific errors (the American peppered moth is not *B. cognataria* but *B. betularia*, the same species as in Britain,

for example), mar the book for biologists.

The biggest shortcoming, however, is Hooper's failure to emphasize that, despite arguments about the precise mechanism of selection, industrial melanism still represents a splendid example of evolution in action. The dramatic rise and fall of the frequency of melanism in *Biston betularia*, occurring in parallel on two continents, is a compelling case of evolution by natural selection. No force other than selection could have caused such striking and directional change. Hooper's grudging admission of this fact occupies but one sentence: "It is reasonable to assume that natural selection operates in the evolution of the peppered moth."

This issue matters, at least in the United States, because creationists have promoted the problems with *Biston* as a refutation of evolution itself. Even my own brief critique of the story (*Nature* 396, 35–36; 1998) has become grist for the creationists' mill. By peddling innuendo and failing to distinguish clearly the undeniable fact of selection from the contested agent of selection, Hooper has done the scientific community a disservice.

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Competition between women

A Mind of Her Own: The Evolutionary Psychology of Women

by Anne Campbell

Oxford University Press: 2002. 402 pp. £21.99, \$40

Elizabeth Cashdan

This provocative book argues that competition among women has been an important, yet largely ignored, force in human sexual selection. Competition between males is usually thought to be more intense than female competition because greater male variance in reproductive success means that men are playing for higher stakes. But Campbell suggests that this is misleading: "The variance may not be as great as between males but that is irrelevant because females are not in competition with males, they are in competition with other females." Mothers (not fathers) are critical to offspring survival, and differences among women in their success in this endeavour have shaped the way that women think, feel and behave.

The book opens with a sharp and satisfying critique of postmodernist biophobia and a skilful rebuttal to those who distrust evolutionary psychology's scientific methods and fear its political implications. It closes with an excellent in-depth account of the evolutionary reasons for individual variation. In between, Campbell shows us how and why women compete.

Women are clearly less physically aggressive and less risk-prone than men, but why? Campbell rejects the belief that it is a "default option that results from lower incentives for competition" and attributes it instead to women's greater parental investment: they have more to lose from violent and risky behaviour. This behavioural difference, she argues, is mediated by a sex difference relating to fear of injury and a neurochemistry that makes women better able to inhibit aggressive impulses.

These arguments are extended in Campbell's discussion of sex differences in dominance and status-seeking. The rewards of high status "are just as great for females as for males — arguably greater because resources fuel the survival of offspring in which they have already invested while for males it merely buys a ticket in the copulatory lottery of possible fatherhood", she argues. What differs, she continues, is not the rewards, but the costs (the risk of injury). This argument explains why women are less prone to seek high status through aggressive competition. It is also used to good effect in a later chapter on women and crime, in which Campbell explains why women commit

fewer and less-violent crimes than men do.

But this fear of injury does not explain why women should actively seek egalitarianism, nor why they should form communal bonds with unrelated individuals that are more enduring and less exchange-orientated than the alliances formed by men. While most of the book is devoted to explaining how and why women compete, Campbell devotes a chapter to this more conciliatory side of female nature. She suggests that their communal tendencies are an evolved response to male philopatry, women's consequent lack of kin support, and the need for protection from men. Perhaps. It is a topic that deserves more attention from evolutionary psychology than it has received.

Many books discuss human sex differences from an evolutionary perspective, but this one differs from most as it is more a monograph than a text. Campbell has a point to make, and she integrates a vast array of material in doing so. That said, the book would nonetheless make a fine text for a graduate course on the evolutionary psychology of women. Among the book's strengths are its emphasis on conditional strategies, its integration of 'ultimate' (adaptive) and 'proximate' (neurological and biochemical) levels of explanation, and its authoritative treatment of female aggression — a field in which Campbell has done considerable ground-breaking research. Notwithstanding its specialized focus, it does cover the bases in

the evolutionary psychology of human sex differences, and the treatment is thorough, thoughtful and up-to-date. The writing is also clear and engaging.

My pleasure in reading the book was repeatedly marred, however, by the publisher's treatment of the references. They are grouped by chapter at the end of the book (rather than being listed alphabetically), and it is a guessing game to find the chapter you need. If references must be grouped this way, please give readers a hint about where to look by indicating the chapter title at the top of each page of references.

Elizabeth Cashdan, *Department of Anthropology, University of Utah, Salt Lake City, Utah 84112-0060, USA.*

Science in culture



Natural style

Ideas taken from science are proving to be fashionable.

David Cyranoski

Like the criss-cross pattern of a sunflower head and the spiral of a nautilus shell, Eri Matsui's best-selling wedding dress (above, far right) can be described using the Fibonacci mathematical sequence. The Japanese fashion designer used increasingly short layers of fabric in the skirt to form sections in a 1, 1, 2, 3, 5, 8, 13, 21, 35, ... pattern, culminating in 56 narrow tufts around the waist. "People recognize the beauty in the order, whether they are looking at the dress or wearing it," says Matsui, who has devoted herself to finding universal forms to make women look beautiful.

Having experimented with images from knot theory, fractal models and Klein bottles, Matsui has more recently been scouring the world of biology for universal forms to use. Her November 2000 collection, for example, with the theme "Brain, Mind, Computer, and Fashion", showcased dresses with colourful images of neurons and others whose thick folds approximate the brain's contours (above in lilac).

Best known in Japan for her somewhat unconventional wedding dresses, Matsui knows that these kinds of design are unlikely to become

a common sight on the streets of Tokyo or in wedding halls. "But these designs help open up the imagination, and provide a basis for designing more practical clothing for everyday wear," she says. Practical clothing based on universal forms does not have to be conventional, she insists.

For her last collection, entitled "A Changing Erotic Lifeless Object", shown on 18 April, Matsui tailored several sets of clothes around cell division and differentiation. In one series, the first model emerged onto the catwalk totally enshrouded in an egg-shaped netting. The next, with netting billowing out above and below a tight waistline, was undergoing the first stage of cell division. The series continued with representations of multiple cell stages. Another series (above, left five images) began with straight horizontal lines representing a single cell that gradually curved, model by model, becoming increasingly individual and specialized. The series ended with a death stage, in which slits represented cell walls breaking apart.

Matsui hopes that her work will help us to realize what we are, and recognize the beauty

within ourselves.

"When people think about the brain or neurons, they think of them as something external, something that scientists tell us about, something to be looked at objectively — not as part of us," she says. "On the other hand, clothes really are lifeless and external — but when we put them on we think of them as part of us." Clothes that depict or represent these biological processes can allow us to experience directly what we are, she adds.

David Cyranoski is Nature's Asian-Pacific correspondent.

Eri Matsui's next show will be held in Tokyo in autumn.

High above the Earth

Henry Rishbeth

We have come far in the 100 years since Oliver Lodge gave the first physical explanation of why Marconi could send radio waves around the curved Earth. Using the new knowledge of electrons and ionization, Lodge realized that solar ultraviolet radiation produces a conducting layer that reflects radio waves. We call this the ionosphere, a term coined by Robert Watson-Watt in 1926 to replace its previous name, the Heaviside layer (after the physicist Oliver Heaviside). In modern terms, the ionosphere is a weakly ionized plasma or electron-ion gas embedded in the thermosphere, the hot, tenuous region above 80 km that comprises the top few millionths of the atmosphere's mass. We are now approaching the stage where we can link our models of the thermosphere and ionosphere with models of the lower atmosphere.

For a century the ionosphere has been used for communications, but it is by no means a constant 'mirror in the sky'. Although its E layer (100–120 km above the ground) and F1 layer (170–200 km) usually behave in a regular, solar-controlled way, the F2 layer (250–350 km) does not. It is this F2 layer, with the greatest density of free electrons, that is potentially the most effective reflector of radio waves. But its variability in height and density, its strange day/night and seasonal behaviour, and its complex response to geomagnetic disturbances have long puzzled scientists and infuriated users of radio communications. This matters because the ionosphere is still widely used

for radio communication; furthermore, the ionosphere can severely affect satellite transmissions that pass through it, causing errors in the Global Positioning System, for example. Ionospheric disturbances can also create strong electric currents in the E layer that can disrupt cable communications and terrestrial electric power systems.

Rockets, satellites and radar have enabled us to build up a comprehensive picture of the upper atmosphere. The ionospheric plasma — being far easier to detect and measure than the neutral atoms and molecules — serves as a 'tracer', the ionospheric measurements acting as 'diagnostics' for the composition, temperature and dynamics of the greatly preponderant neutral air.

Like the lower atmosphere, the thermosphere is a heat engine on a global scale, driven by radiation from the Sun. Whereas the lower atmospheric engine is driven by infrared and visible radiation, the thermosphere is driven by short-wavelength ultraviolet and X-radiation which heats, dissociates and ionizes the air. It is also driven by the energy input from the particle stream known as the solar wind, which shapes and controls the Earth's magnetic envelope — the magnetosphere — and deposits heat in the auroral zones that surround the Earth's magnetic poles.

We model the workings of this 'engine' using computational 'global circulation models'. To generate these models, the equations that embody the principles of conservation of mass, momentum and energy, for electrons and for many types of ion and neutral particle, must be solved on a grid of latitude, longitude and height for every minute of the day, and the results validated by many measurements — no mean feat! Like their meteorological counterparts for the lower atmosphere, these models are indispensable for describing, explaining and ultimately forecasting the behaviour of this complex system.

The picture that emerges is one in which the ions in the F2 layer are mostly atomic oxygen (O^+), and their recombination with electrons involves reactions with neutral molecules (O_2 and N_2). For this reason, the ion and electron concentrations depend on the atomic/molecular ratio of the thermospheric air. This ratio, which is mainly controlled by solar radiation, is seasonally modified by the vertical and horizontal winds of the global circulation. The picture indicates the summer-to-winter flow of air in the thermosphere, and also the flows driven by auroral heating (the return flows at lower heights and through the night side are not shown). The circulation is distorted during geomagnetic disturbances, when the auroral zones become more active and move to lower latitudes.

Ionosphere

For a century, the strange and variable behaviour of this 'mirror in the sky' has puzzled scientists and infuriated users of radio communications.

These ideas help us to solve long-standing puzzles, such as why electron density in the F2 layer peaks in winter over Europe, North America and Australasia, but at the equinoxes at low latitudes and in the South Atlantic. Another tricky question is why electron density is usually drastically reduced (but is sometimes increased) during geomagnetic storms. Investigating these problems helps to validate the models and to make sense of the vagaries of radio propagation.

Not all ionospheric variability can be blamed on solar or geomagnetic disturbances. Some may originate from below, caused by waves and tides transmitted up to the ionosphere. So does lower-atmosphere 'weather' affect the ionosphere, or vice versa? Our linked circulation models, embracing the thermosphere and the lower atmosphere, include the interchange of momentum and energy carried by radiation, wind and waves.

The ultimate prize is a complete top-to-bottom, predictive model of the Earth's atmosphere that incorporates our rapidly advancing knowledge of Sun–Earth relationships and the magnetosphere. Running such a model on timescales of days, hours or even minutes should enable us to make better forecasts of the 'space weather' that affects spacecraft and communications. On timescales of years and decades, the models should give us a better understanding of solar and upper-atmospheric influences on climate and global change. There are many side questions, such as whether and how the ionosphere is affected by tropical storms, volcanic activity or earthquakes? All this may sound visionary, but Lodge, a visionary of his day, would surely have approved.

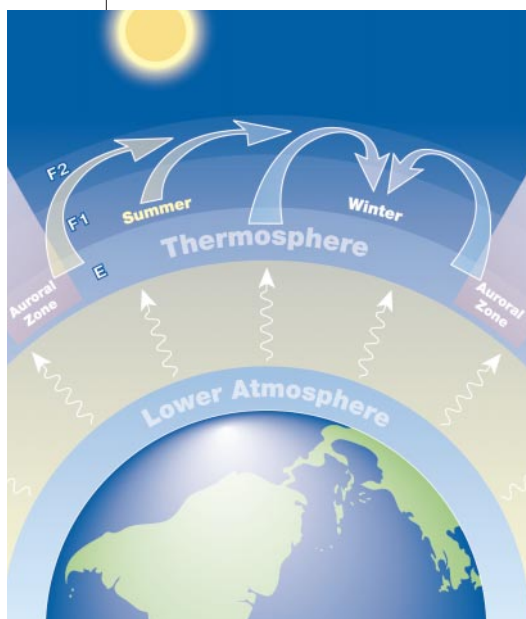
Henry Rishbeth is emeritus professor in the Department of Physics and Astronomy, University of Southampton, Southampton SO17 1BJ, UK.

FURTHER READING

- Lodge, O. *Nature* **66**, 222 (1902).
 Hargreaves, J. K. *The Solar–Terrestrial Environment* (Cambridge Univ. Press, 1992).
 Roble, R. G. *Am. Geophys. Un. Monogr.* **123**, 53 (2000).
 Schunk, R. W. & Nagy, A. E. *Ionospheres: Physics, Plasma Physics and Chemistry* (Cambridge Univ. Press, 2000).
 Gardner, G. W. *Nature* **224**, 1096 (1969).
www.wdc.rl.ac.uk/ionosondes/ionosondes.html

See '100 years ago', *News and Views*, p. 26

VICKY ASKEW



Stem-cell competition

Stuart H. Orkin and Sean J. Morrison

The debate continues over the relative merits of using embryonic and adult stem cells for research — and perhaps, one day, to treat patients. Two new papers look at the abilities of these remarkable cells.

Last August tiny cells in Petri dishes captivated primetime television audiences. In a carefully worded address, US President George Bush discussed the medical potential, risks and ethics of studying human embryonic stem (ES) cells. For proponents, these cells represent our greatest hope for treating devastating disorders such as Parkinson's disease, diabetes and spinal-cord injuries. But for those who are adamantly opposed to the use of cells derived from human embryos, stem cells from adults have been advocated as an ethically palatable and experimentally reasonable alternative.

Two papers in this issue rekindle the debate. On page 50, Kim and colleagues¹ describe how they generated a specific class of neurons from cultured mouse ES cells and used the neurons to reverse symptoms of Parkinson's disease in rats. In other words, ES cells can generate specialized cell types that are therapeutically effective in animals. Meanwhile, Jiang and colleagues² (page 41) have derived remarkably versatile cells from the bone marrow of adult mice, rats and humans. These two studies further the promise of stem cells even while they stoke the debate about whether such cells should be obtained from embryos or adults.

Stem cells, essential building blocks of multicellular organisms, have two defining properties — they can produce more stem cells and they can generate specialized cell types such as nerve, blood or liver cells. Stem cells come in different varieties, relating to when and where they are produced during development, and how versatile they are. Pluripotent stem cells give rise to all cell types. The best characterized are ES cells (Fig. 1), which are derived from very early mouse or human embryos. These cells proliferate indefinitely in culture, while retaining the potential to differentiate into virtually any cell type when coaxed. So, in principle, ES cells might be able to generate large quantities of any desired cell for transplantation into patients.

Stem cells collected from tissues of adults or older embryos are typically more restricted in their developmental potential and ability to proliferate. For example, blood-forming (haematopoietic) stem cells make all types of blood cells *in vivo*, but proliferate little in culture and have been thought not to make cells of other tissues. Recent studies have raised the possibility that some adult

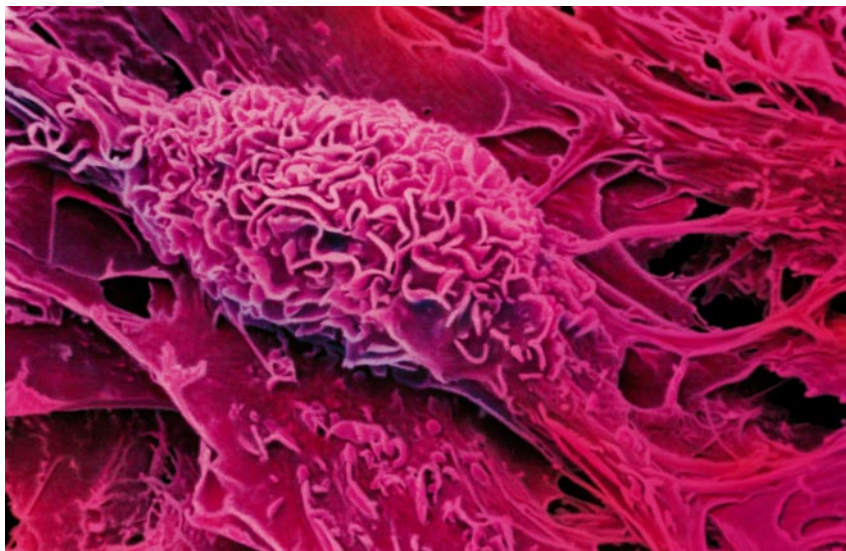


Figure 1 **Embryonic stem cell — primetime exposure.**

stem cells can give rise to cells outside their tissue of origin; however, these results are controversial³ and have often proved difficult to reproduce⁴. Nonetheless, those opposed to using human ES cells tout the possibility of pluripotent adult stem cells as a way of realizing medical gain without ethical pain. Although researchers generate ES cells from pre-implantation embryos in culture, and several countries have sanctioned deriving human ES-cell lines from 'surplus' embryos created through *in vitro* fertilization, some remain uncomfortable with the destruction of human embryos, even those destined never to be implanted in a uterus.

Beyond these ethical issues, there are technical obstacles to the use of ES cells. First, these cells can be obtained only from very early embryos and, although several human ES-cell lines have been made, they will not be immunologically compatible with most patients who require cell transplants. So researchers will need either to derive many more ES-cell lines or to customize ES cells on a patient-by-patient basis by 'therapeutic cloning'. Second, undifferentiated ES cells form teratomas — benign tumours containing a mixture of tissue types — after being transplanted. Thus ES cells must be reliably differentiated into the appropriate cell type in culture before transplantation.

Moreover, until now it had not been proved that specialized cells derived from

cultured ES cells can actually function within tissues after transplantation^{5,6}. For example, mouse ES cells produce insulin-secreting cells in culture, but such cells have not been shown to reverse high blood sugar levels in mice with symptoms of diabetes⁶. It is perhaps not surprising that cells generated *in vitro* might not be equivalent to those arising *in vivo*, given the extensive cellular interactions and 'education' that take place during development. But Kim *et al.*¹ have now overcome this problem in their work on rats with symptoms of human Parkinson's disease.

Parkinson's disease is caused by the death of neurons, found in the striatal region of the brain, that produce the neurotransmitter dopamine and are involved in controlling movements. The symptoms of Parkinson's disease have been successfully treated in animals by transplanting dopamine-producing neurons into the striatum to replace lost neurons. In humans, however, results have been mixed⁷. A central problem has been the scarcity of suitable neurons. These neurons would be abundant if they could be produced from cultured ES cells, but this has proved inefficient and it has been uncertain whether the resulting neurons would be effective.

Enter Kim *et al.*¹. To enhance the yield of dopamine-producing neurons, the authors expressed the Nurr1 protein — which is needed to generate these neurons *in vivo* — in cultured ES cells. The resulting neurons survived

YORGOS NIKAS/SPL

NATURE

100 YEARS AGO

Mr. Marconi's Results in Day and Night Wireless Telegraphy. I can assure

Prof. Joly that his explanation will not do. The observed effect, which if confirmed is very interesting, seems to me to be due to the conductivity, and consequent partial opacity, of air, under the influence of ultra-violet solar radiation. No doubt electrons must be given off from matter (dust as well as other matter) in the solar beams; and the presence of these will convert the atmosphere into a feeble conductor. Conducting power in the sea-water surface assists and guides the waves, retaining them in two dimensions after the same fashion as a telegraph wire retains them in one; but conductivity in the dielectric itself will tend to dissipate and enfeeble the waves, by a process of reflection resulting in some amount of distortion.

O. Lodge

From *Nature* 3 July 1902.

50 YEARS AGO

During the past three years, experiments have been conducted with subjects who have been given the task of solving mental-test items of a particular type. Suppose P_c be the probability that a particular subject will continue to work at a particular problem for some time t before giving it up, and let P_e be the probability that if a solution is recorded within this time it will be the correct one. If the universe of discourse is now restricted to correctly solved items, the probability (P_s) that a particular correct solution will be returned within a period of t sec. after the moment the problem is presented will be a function both of the dynamics of the problem-solving process as such and also of P_c Let P_s be defined as the probability which would obtain if $P_c = 1$ for $t = \infty$. No comprehensive statement can be made about a person's ability to solve a problem which does not involve at least these three probabilities, that is, any attempt to measure 'intelligence' by mental-test methods should involve assessments of P_c , P_e and P_s D is the difficulty of the problem being considered, defined in the conventional but arbitrary fashion $D = [100 - R]$, and R is the percentage of an adult British population (unselected) who achieve success with the item.

From *Nature* 5 July 1952.

Property	ES cells	MAPCs
Origin	Embryo	Adult bone marrow
Growth potential	Indefinite	Indefinite
Differentiate into most cell types?	Yes	Yes
Require growth factor LIF?	Yes	Yes (for mouse MAPCs)
Stability of chromosome integrity	Reasonably stable	High
Expression of marker protein Oct-4	High	Very low
Contribute to germ cells?	Yes	Not known
Produce blood cells on transplantation?	No	Yes
Display dosage compensation?	No	Not known
Efficiency of gene modification	High	Not known
Possibility of autotransplantation?	No	Yes

Figure 2 Comparison of embryonic stem (ES) cells, and the multipotent adult progenitor cells (MAPCs) described by Jiang *et al.*². ES cells (and mouse MAPCs) need LIF for growth; other cultured cells do not, so this seems to be a unique feature of pluripotent stem cells. The expression of Oct-4 in ES cells correlates with their versatility; if MAPCs are similar to ES cells one might expect comparable expression of Oct-4. Germ cells are eggs or sperm. Dosage compensation inactivates one X chromosome in females, so that males (which have one X and one Y chromosome) and females (XX) express X-chromosome genes to the same degree. Autotransplantation refers to the possibility of taking stem cells from patients, deriving the required specialized cells, and transplanting them back in the patients.

after being transplanted into rats with symptoms of Parkinson's disease, and showed appropriate electrophysiological properties. More impressively, the rats started to recover normal movements. Others have transplanted partly differentiated ES cells to ameliorate Parkinson's symptoms in rats, but observed tumours in some of the animals⁸. By demonstrating efficacy while avoiding tumour formation, Kim *et al.* have achieved a proof of principle, although ES cells that have been genetically modified in this way might not be desirable for use in people.

To sidestep the disadvantages of ES cells, it would be ideal to identify a pluripotent adult stem cell that proliferates indefinitely in culture. This is just what Jiang *et al.*² seem to have done. Prior work⁹ indicated that there is a population of stem cells in bone marrow, known as mesenchymal stem cells, that can form muscle, cartilage, bone and fat. Taking a similar approach, Jiang *et al.* started with non-haematopoietic bone marrow cells, cultured them, and isolated a population that they called multipotent adult progenitor cells (MAPCs).

In culture, single mouse MAPCs proliferated indefinitely and differentiated into many specialized cell types. Upon injection into mouse blastocyst-stage embryos, individual MAPCs contributed widely to developing tissues. And when injected intravenously into adult mice they gave rise to several blood and epithelial cell types. These stunning findings cannot readily be explained by incorrect identification of the progeny of the transplanted stem cells. But it remains possible that the MAPCs might have fused with blastocyst cells^{10,11}, explaining their versatility in that experiment.

Are MAPCs the adult equivalent of ES cells? More data are needed, yet so far it seems that there are both similarities and

important differences between these cells (Fig. 2). What, then, are MAPCs? They might be very rare pluripotent stem cells that persist from the embryo into adult life. To prove this it would be necessary to identify these cells prospectively *in vivo* (rather than retrospectively *in vitro*) by the marker proteins they express, and to purify them without an intervening culture step.

Alternatively, MAPCs might not actually exist *in vivo*. The extended period of culture might have triggered certain bone marrow cells to regress to a more primitive state, just as primordial germ cells — from which eggs and sperm are produced — can be reprogrammed in culture to acquire properties like those of ES cells^{12,13}. (In fact, ES cells do not exist as such in embryos; they arise after being cultured.) If so, we have much to learn about how cells can be reprogrammed in culture.

MAPCs might prove useful in treating diseases irrespective of their origin. But, until they have been identified *in vivo*, it's premature to speculate that they might have a natural role in repairing injured tissues. Moreover, although MAPCs can generate many specialized cell types, it remains to be seen whether those cells function normally and could be used to treat animals, as Kim *et al.*¹ used ES-cell-derived neurons. Such experiments will now be a priority, given the possibility of isolating MAPCs from the bone marrow of any patient, and transplanting the progeny of these cells back to the patient without risk of rejection.

The potential benefits of treating diseases by using specialized cells generated *in vitro* are enormous. But much further work is needed. Possible risks include the tendency of ES cells to form teratomas, and the unknown hazards of using cells — whether ES cells or MAPCs — that have been cultured

for long periods. And, although MAPCs seem to have normal chromosomes, it is important to establish that the pathways governing cell proliferation are unperturbed. Otherwise, short-term gains might fall prey to long-term complications.

The work of Kim *et al.*¹ and Jiang *et al.*² will not resolve the debate over embryonic versus adult stem cells. Rather, it underscores the need for research in this area to continue unfettered by political concerns. Only then will the public have a chance to get what it deserves: novel, validated and safe treatments for intractable diseases. ■

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1. Kim, J.-H. *et al.* *Nature* **418**, 50–56 (2002); advance online publication, 20 June 2002 (doi:10.1038/nature00870).
2. Jiang, Y. *et al.* *Nature* **418**, 41–49 (2002); advance online publication, 20 June 2002 (doi:10.1038/nature00900).
3. Wells, W. J. *Cell Biol.* **157**, 15–18 (2002).
4. Morshead, C. M., Benveniste, P., Iscove, N. N. & van der Kooy, D. *Nature Med.* **8**, 268–273 (2002).
5. Kyba, M., Perlingiero, R. C. R. & Daley, G. Q. *Cell* **109**, 29–37 (2002).
6. Lumelsky, N. *et al.* *Science* **292**, 1389–1394 (2001).
7. Freed, C. R. *et al.* *New Engl. J. Med.* **344**, 710–719 (2001).
8. Bjorklund, L. M. *et al.* *Proc. Natl Acad. Sci. USA* **99**, 2344–2349 (2002).
9. Pittenger, M. F. *et al.* *Science* **284**, 143–147 (1999).
10. Ying, Q.-Y., Nichols, J., Evans, E. P. & Smith, A. G. *Nature* **416**, 545–548 (2002).
11. Terada, N. *et al.* *Nature* **416**, 542–545 (2002).
12. Matsui, Y., Zsebo, K. & Hogan, B. L. M. *Cell* **70**, 841–847 (1992).
13. Donovan, P. J. *Curr. Top. Dev. Biol.* **29**, 189–225 (1994).

Planetary science

An older face for Mars

Sean C. Solomon

Mars has a north–south divide in the age of its surface, as judged by the density of impact craters. Altimetry data, which by inference provide a subsurface view of the planet, reveal that the divide is only skin deep.

A surprising finding from the exploration of Mars by orbiting spacecraft in the 1970s was that the southern and northern hemispheres have very different surfaces. The density of impact craters seen in images taken by Mariner 9 and Viking Orbiter indicated that the surface of the topographically high southern hemisphere is old enough to have preserved the effects of the early, heavy impact bombardment of the inner Solar System (known from lunar studies to have occurred before about 3.7 billion years ago). The surface of the northern hemisphere, in contrast, was revealed to be generally lower in elevation, to consist of smooth plains, and to contain a far lower density of impact features — implying a surface age hundreds of millions to billions of years younger.

A new view of this striking hemispherical contrast, the Martian ‘crustal dichotomy’, has come from an analysis of observations collected by the Mars Global Surveyor spacecraft, which has been in Martian orbit since 1997. From subtle signatures in topography measured by the Mars Orbiter Laser Altimeter Experiment¹, Frey and others² have identified a large population of nearly buried impact structures in both hemispheres that were not evident in the spacecraft images. More importantly, the areal density of these features, together with those previously mapped, indicates that the northern hemisphere has a buried surface that is essentially as old as the surface of the southern uplands.

Frey and colleagues² took the following approach in their search for buried impact features. From regional altimetric maps, in which different colours were assigned to

narrow intervals of elevation from a global gridded data set, the group catalogued all roughly circular, localized depressions. They identified such depressions as candidate impact features if concentric segments of contours collectively totalled at least 180° of arc, and if the preserved relief exceeded 50 m — a figure much larger than the altimetry accuracy¹ of about a metre. Many of the features so catalogued had been identified as impact craters from orbiter images. As shown in Fig. 1, however, many others had not. In particular, Frey *et al.* counted 644 potential impact features greater than 50 km in diameter in the northern lowlands, many more than the 90 such features discernible from Viking Orbiter images. A strong argument that most, if not all, of the newly identified features are largely buried impact craters is that their areal density as a function of their diameter has the same form as the distribution for known impact structures. The southern highlands also contain buried impact craters newly recognized by this method, but they do not increase the total population of impact structures by as large a factor as in the north.

Frey and colleagues’ main conclusion is that the geologically younger units constituting the uppermost crust of the northern hemisphere are a relatively thin veneer of material that incompletely masks an underlying ancient surface. There were a few previous clues that this was so. Careful photographic mapping led to the suggestion³ that underlying the deposits in the Utopia

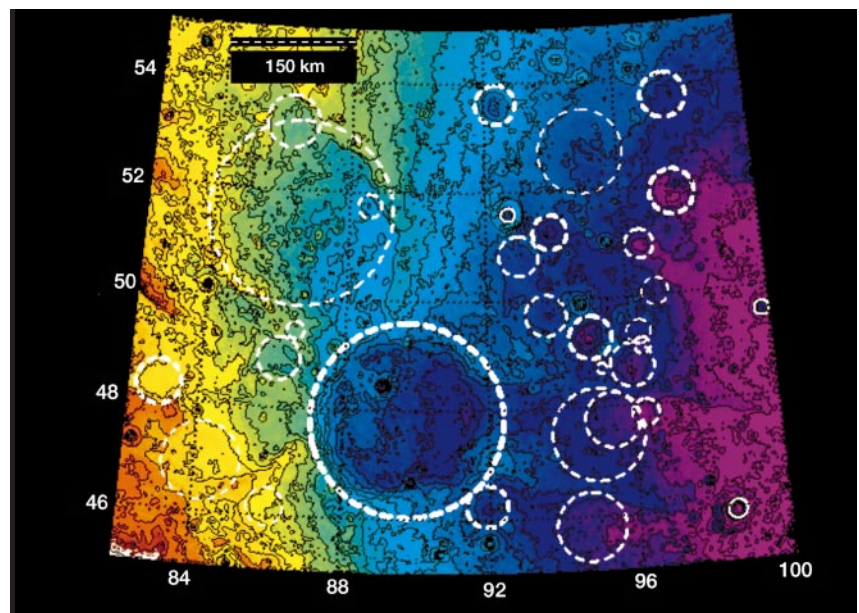


Figure 1 Partly buried impact craters, identified by Frey *et al.*², in the northern lowlands of Mars. The part of the planet shown is near the Utopia region. Elevation is colour-coded, with purple grading through blue and green to yellow, orange and red as height increases (orthographic projection, 50-m contour interval). Craters greater than 15 km in diameter visible in the earlier Viking Orbiter images are circled by solid lines. The many more craters discernible only from their topographic signatures are shown by thick dashed lines; thinner dashed lines denote less confident identifications.

H. V. FREY AND J. H. ROARK

region of the northern plains is a 3,300-km impact basin, a feature so large that it must date from the early heavy bombardment. That hypothesis was strengthened by the discovery of a broad depression, the outline of which coincides with that of the buried basin⁴, and by a large, positive, gravity anomaly having a magnitude consistent with the nearly complete infilling of an ancient impact depression by younger sedimentary and volcanic material⁵. But the work of Frey *et al.* extends the mapped area of ancient surface to nearly the entire northern lowlands and quantifies the density of impact features.

The crustal dichotomy, then, might be the most ancient structural feature preserved on the Martian surface. If so, there are several implications for our understanding of the geological and geophysical evolution of the planet. The northern lowlands must have exerted a primary influence on the flow direction of surface and subsurface water on Mars for the entire duration of preserved geological history⁶, and much of the material making up the veneer overlying the ancient northern surface probably consists of sediments. The high density of newly detected large impact structures within the Utopia basin — whose original depth might have been comparable to the nearly 10-km relief of Hellas, a partly filled basin of similar diameter in the south — implies that much of the resurfacing of the northern hemisphere occurred during the period of early impact bombardment. The incomplete burial of impact craters allows the thickness of the younger northern-plains units to be estimated at typically 1–2 km, but that figure could exceed 5 km in a few areas near major volcanic centres where no older craters can be seen². The difference in elevation between the north and the south requires a generally thinner crust in the north⁵. Because lateral flow of lower crust would tend to reduce such variations if the temperature at the base of the crust exceeded about half the local melting temperature^{5,7}, preservation of the dichotomy implies that the lower crust cooled rapidly after early crustal formation — perhaps as a result of deep hydrothermal circulation⁸.

A question, first raised when the crustal dichotomy was discovered, remains. What caused it? Suggestions have ranged from removal of crust in the northern hemisphere by one or more large impacts, to hemispherical differences in crustal addition or internal recycling by dynamical processes in the underlying mantle. None has proved fully persuasive⁹. At the least, however, the demonstration that the crust in both hemispheres formed very early in the history of Mars adds a fresh requirement for any successful explanation of this long-standing mystery. ■

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1. Smith, D. E. *et al.* *J. Geophys. Res.* **106**, 23689–23722 (2001).
2. Frey, H. V., Roark, J. H., Shockey, K. M., Frey, E. L. & Sakimoto, S. E. *H. Geophys. Res. Lett.* **29**, 10.1029/2001GL013832 (2002).
3. McGill, G. E. *J. Geophys. Res.* **94**, 2753–2759 (1989).

4. Smith, D. E. *et al.* *Science* **284**, 1495–1503 (1999).
5. Zuber, M. T. *et al.* *Science* **287**, 1788–1793 (2000).
6. Baker, V. R. *Nature* **412**, 228–236 (2001).
7. Nimmo, F. & Stevenson, D. J. *J. Geophys. Res.* **106**, 5085–5098 (2001).
8. Parmentier, E. M. & Zuber, M. T. *Lunar Planet. Sci.* **33**, 1737 (2002).
9. McGill, G. E. & Squyres, S. W. *Icarus* **93**, 386–393 (1991).

Embryology

Fluid flow and broken symmetry

Claudio D. Stern

The asymmetries between the right- and left-hand sides of the body are initiated at an early stage of development. Two groups provide welcome news of progress in revealing the mechanism concerned and its generality.

How an embryo first distinguishes its left from its right side has baffled embryologists for a long time. The rotational beating of cilia — hair-like structures attached to individual cells — is known to be essential for the process. But cilia have been seen only in mouse embryos, and it has remained unclear whether their movement could really generate the necessary molecular asymmetries. Papers by Essner *et al.*¹ and Nonaka *et al.*² (pages 37 and 96 of this issue) set our understanding on a much firmer footing in both respects.

Despite its superficial appearance of bilateral symmetry, the vertebrate body plan is asymmetric in several respects, most obviously in the position of internal organs such as the heart and parts of the gut. Left–right asymmetry first arises in the embryo at around the stage — the gastrula — when the three major cell layers of ectoderm, mesoderm and endoderm are first laid down. But until recently we knew virtually nothing about the molecular mechanisms responsible.

The turning point came in 1995 when four genes (*Sonic hedgehog*, *Nodal*, *HNF3 β* and the *Activin-receptor IIA*) were identified as being expressed on one or the other side of the chick embryo at the gastrula stage, and their activities were implicated in heart turning³. However, subsequent work revealed that only one of these, *Nodal*, is expressed asymmetrically in all vertebrates. Shortly afterwards it was discovered that a mouse mutant, called *iv* and characterized by random positioning of internal organs, carries a mutation that inactivates left–right dynein (LRD), a protein required for the beating of cilia⁴. Researchers then looked for cilia in the mouse gastrula and found that the ‘node’, a critical organizing structure in the midline of the early embryo, does indeed possess short cilia protruding from its cells, which beat in an anticlockwise circular motion and generate a leftwards flow of fluid that is strong enough to displace solid particles to the left^{5,6}. But again, the cilia could be found only in the mouse. Could different vertebrates

have evolved different ways of establishing asymmetry? And could the beating of cilia really be sufficient to generate molecular asymmetry by removing a ‘morphogen’ signal from one side of the embryo and enriching it on the other?

The papers by Essner *et al.*¹ and Nonaka *et al.*² answer both questions. Essner *et al.* reveal that cilia, as well as LRD, are indeed present in all the major vertebrate groups at appropriate stages and locations to generate left–right asymmetry. Nonaka *et al.* show that a flow of fluid in the reverse direction to that generated by cilia can randomize embryonic asymmetry — and that artificially induced fluid flow is enough to control the position of the internal organs in *iv* mutant mice.

The idea that the circular beating of tiny cilia could be enough to generate a biologically meaningful molecular gradient within a large, relatively open fluid space seemed rather unlikely when it was first proposed⁵. Nonaka and colleagues have designed an ingenious mechanical device to demonstrate that it is indeed possible to control the distribution of molecules by regulating the direction of fluid flow around embryonic cells. The experimental design was similar to one described nearly 20 years ago⁷. There, it was demonstrated that gentle flow applied to a wounded sheet of cells in culture restricted healing growth of the wound to the downstream side of the flow, as if it were controlled by the local distribution of a growth factor. The study by Nonaka *et al.* now reveals a likely role for this mechanism *in vivo*. It highlights the significance of bio-mechanical phenomena in generating biological pattern, an idea that has hitherto been broadly dismissed.

In contrast, the finding¹ that cilia and LRD are associated with the node or its equivalent structures in all major vertebrate groups is reassuring. Developmental biologists generally choose one ‘model’ organism or another according to the experimental advantages it offers, with the underlying assumption that the basic principles should

be much the same in different species. That this is generally true is evident in a huge body of data revealing that genes responsible for patterning the body of the fruitfly have similar functions in vertebrates.

Several questions remain open. What, for instance, are the critical molecules whose distribution is being controlled by ciliary beating? What mechanisms determine the direction of ciliary rotation within each ciliated cell? Why is *Nodal* the first asymmetrically expressed gene common to all vertebrates, whereas other factors are variable? And in the chick embryo, the earliest molecular asymmetry seems to be the right-sided localization of the *Activin-receptor IIA*³ just before the earliest expression of *LRD* and the appearance of nodal cilia¹, which raises the question of whether other symmetry-breaking mechanisms might exist at earlier stages of development. ■

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1. Essner, J. J. *et al. Nature* **418**, 37–38 (2002).
2. Nonaka, S., Shiratori, H., Saijoh, Y. & Hamada, H. *Nature* **418**, 96–99 (2002).
3. Levin, M., Johnson, R. L., Stern, C. D., Kuehn, M. & Tabin, C. *Cell* **82**, 803–814 (1995).
4. Supp, D. M., Witte, D. P., Potter, S. S. & Brueckner, M. *Nature* **389**, 963–966 (1997).
5. Nonaka, S. *et al. Cell* **95**, 829–837 (1998).
6. Okada, Y. *et al. Mol. Cell* **4**, 459–468 (1999).
7. Dunn, G. A. & Ireland, G. W. *Nature* **312**, 63–65 (1984).

Materials science

Crystallization of silicon ideas

John Robertson

The more desirable form of silicon for use in display screens is also the more expensive to manufacture. Understanding how crystalline silicon forms could be a key to cheaper communications devices.

Amorphous silicon is the leading electronic material for large-area applications, used in solar cells and in the thin-film transistors that make up liquid-crystal displays. However, amorphous silicon suffers from an electrical instability that causes a gradual loss of conversion efficiency in solar cells. There is another form of silicon, nanocrystalline silicon, that is more stable and whose charge-carriers (electrons and holes) are more mobile, making it more attractive for use in these applications.

Amorphous silicon can be made easily by deposition from a silane (SiH_4) plasma, and nanocrystalline silicon can be made by the same process, under slightly modified conditions. But the exact means by which nanocrystalline silicon, rather than amorphous silicon, forms has been the subject of debate. Now Sriraman and colleagues¹ might have found the answer: on page 62 of this issue, they propose that hydrogen atoms from the silane plasma catalyse the

rearrangement of Si–Si bonds, triggering a solid-state transformation of the random amorphous-silicon network into the more ordered network of nanocrystalline silicon.

Nanocrystalline silicon can be deposited from a silane plasma that has been heavily diluted with hydrogen so that there is a high concentration of atomic hydrogen in the plasma. The most popular explanation of the deposition process has been that nanocrystalline silicon and amorphous silicon are deposited simultaneously, and then the atomic hydrogen etches away amorphous silicon more quickly, leaving mostly nanocrystalline silicon². Once formed, the two solid phases can then only interconvert via the gas phase.

An alternative explanation is that atomic hydrogen permeates the film of amorphous silicon, lowering the energy barriers to the rearrangement of the silicon network into a more stable, more ordered nanocrystalline lattice³. But, until now, the precise mecha-

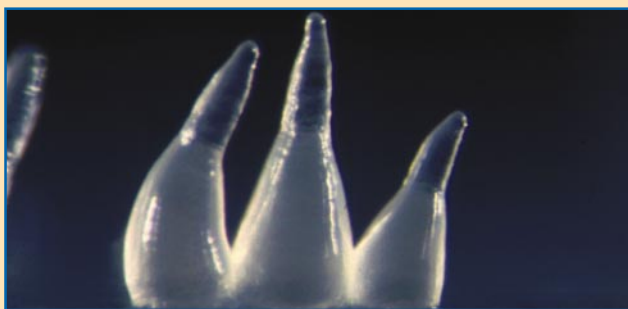
Genome sequencing

Stick it in the family album

You may feel that you have some relations that resemble the cellular slime mould *Dictyostelium discoideum*, shown here. If so, you would be right. For years there has been debate about where this organism sits on the tree of life, and whether it belongs with plants or animals. But a partial genomic sequence — that of chromosome 2, described by Gernot Glöckner *et al.* in this issue (*Nature* **418**, 79–85; 2002) — confirms its closer kinship with animals.

Dictyostelium — *Dicty* to its friends — is a soil amoeba but nonetheless a eukaryote: an organism with a membrane-bound nucleus. It has long been recognized as an excellent model organism. It shows much of the genetic flexibility of yeast, and has complex signalling pathways as well as chemical-sensing behaviours like those seen, for instance, in white blood cells.

A multinational team of sequencing centres (www.uni-koeln.de/dictyostelium/consortium.shtml) is sequencing the roughly 34 million bases of the *Dicty* genome and mining them for insights into eukaryotic life. But sequencing is dogged by the problem of high A–T base-pair content, and subsequent analysis is also challenging because of the high repetitive content (around 10%).



To surmount some of the hurdles in sequencing, the various groups constructed libraries from each chromosome separately and carried

out shotgun sequencing on each library. But the chromosomal libraries are only about 60% pure, so each group is also generating a whole-genome shotgun. By pooling the data from several libraries, Glöckner *et al.* could fish for the maximum number of pieces of chromosome 2 using known chromosome-2 genes as bait. Tying these pieces together and fitting them onto a backbone of clone-based sequences yielded a mostly complete chromosome sequence, which at 8.1 million bases

is about 25% of the genome.

Analysis of chromosome 2 indicates that there are 2,799 protein-coding genes and 73 for transfer RNA. On this basis, the entire genome should contain 10,500 to 11,500 genes, with a density similar to that in budding and fission yeasts. Comparison with sequenced eukaryotic genomes revealed about 45% matches to protein-coding genes, and 55% unique to *Dicty*. Although this number seems high, it is in line with estimates for the available sequences of other eukaryotes.

At the same time, analysis of chromosome 2 has already uncovered more family resemblance, as it encodes proteins similar to several cytoskeleton-related and signalling proteins in animals.

With sequences of the remaining five chromosomes coming shortly, *Dicty* will soon need a page in the family photo album.

Chris Gunter

R. KAY/MRC

nism for this process — given the rather unappealing name of ‘chemical annealing’ — has never been fully specified.

Sriraman *et al.*¹ show how chemical annealing occurs. By substituting deuterium (heavy hydrogen, an isotope with a neutron as well as a proton in the nucleus) for hydrogen atoms, they were able to use the distinctive signature of deuterium in infrared spectroscopy to track the movements of these atoms as they enter the network of amorphous silicon from the plasma. They saw that the hydrogen atoms take up positions at the centres of Si–Si bonds. These Si–H–Si bonds are a high-energy configuration and, according to molecular-dynamics simulations, they decay into various lower-energy states, the most favoured being the crystalline state. Thus, an ordered network of Si–Si bonds forms, and the released hydrogen atoms diffuse away.

This work adds to our knowledge of the role of hydrogen in materials like amorphous silicon, or more strictly ‘hydrogenated amorphous silicon’. The hydrogen atoms are more mobile than the silicon atoms, and the hydrogen content (5–10% in hydrogenated amorphous silicon) is known to tie off or passivate any broken silicon bonds (called dangling bonds). This hugely beneficial effect makes hydrogenated amorphous silicon a viable electronic material. But the mobility of hydrogen atoms can also be a problem. In hydrogenated amorphous silicon under illumination, or in a thin-film transistor turned on for a long time, the movement of hydrogen atoms causes Si–Si bonds to break, creating dangling bonds. This causes a reduction in solar-cell efficiency, or a shift of the electrical characteristics of a transistor. In contrast, the ease of movement of hydrogen in a silicon film helps to form nanocrystalline silicon; the trick is exploiting the hydrogen to achieve the nanocrystalline structure, and then getting rid of as much hydrogen as possible from the final film to improve its stability.

Plasma deposition is the only practical method of making nanocrystalline silicon over the large areas needed for solar cells, although the process is currently limited commercially by low growth rates. (Plasma-deposited nanocrystalline silicon nucleates on the bottom layer and then the grains grow upwards in a competing manner as the film thickens.) A better understanding of the growth process, coupled with the findings of Sriraman *et al.*, will help to optimize the trade-off between higher growth rates and maintaining crystalline quality.

But more important than the manufacture of solar cells is the rapidly growing use of liquid-crystal displays in electronic communications — for laptop and desktop computer monitors, personal digital assistants and cellphones. In most current display technologies, amorphous-silicon transistors

at each pixel store the picture information. Although amorphous silicon is unstable, the pixel transistors are only switched on for a very short time, so their effective lifetime is artificially long. Other components in the liquid-crystal display known as driver transistors are usually mounted in integrated circuits at the edge of a display plate. But there is a trend to mount driver transistors directly on the display plate, as this reduces the cost and complexity of the display. Driver transistors are generally switched on continuously, so they must be stable⁴. Hence, nanocrystalline silicon is required for this purpose.

Nanocrystalline silicon for displays is currently made by laser crystallization of amorphous silicon. Lasers are expensive, but, so far, this is the only feasible method. Could plasma deposition be used instead?

Certainly the insight provided by Sriraman *et al.*¹ suggests this is a promising avenue to explore. There are still issues to be addressed, such as control of the size and orientation of the nanocrystalline grains, but the easy manufacture of stable transistors by plasma deposition would have a major impact on the display-technology industry. ■

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1. Sriraman, S., Agarwal, S., Aydil, E. S. & Maroudas, D. *Nature* **418**, 62–65 (2002).
2. Tsai, C. C., Anderson, G. B. & Wacker, B. *J. Non-Cryst. Solids* **114**, 151–153 (1989).
3. Shirai, H., Hanna, J. & Shimizu, I. *Jpn. J. Appl. Phys.* **30**, L679–L682 (1991).
4. Wehrspohn, R. B., Powell, M. J., Deane, S. C., French, I. D. & Roca i Cabarrocas, P. *Appl. Phys. Lett.* **77**, 750–752 (2000).

Planet formation

Enigmatic emission

Takeshi Oka

The detection of infrared emission from the dusty disk around a distant star signals the presence of the H_3^+ ion. The finding may provide a vital clue to how hydrogen and helium condense into gas-giant planets.

Although there is some consensus on how stars form from interstellar matter, how planets form is less certain. Observations of infrared and radio emissions from dust in protostars have established the presence of protoplanetary disks — regions where dust particles accumulate to form rocky planets like the Earth. But it’s a different story for giant planets like Jupiter and Saturn. How the primordial gas of hydrogen and helium in the region condenses to form giant planets is still a mystery, although it is generally believed that the condensation occurs within a short period.

On page 57 of this issue, Brittain and Rettig¹ report their observations of a protoplanetary disk around the star HD 141569, 320 light years from Earth, in which a giant planet might be in the process of forming. Particularly startling is the detection of strong emission of the hydrogenic ion H_3^+ , which, according to one model, might originate from a gas-giant protoplanet. If the findings are confirmed, Brittain and Rettig have discovered a new astronomical object, opening up a new avenue for the study of the formation of giant planets.

A crucial question is whether protoplanetary disks contain sufficient raw material, H_2 and He, to form Jupiter-like planets. Observations have been controversial, even on this most fundamental issue. Zuckerman, Forveille and Kastner² observed radio emission from CO molecules in the disks surrounding many young stars that are considered to be going through the period of

planet formation; assuming the standard ratio of H_2 to CO (about 10^4), they concluded that the amount of H_2 available was far short of that needed for the formation of a giant planet. However, using data from the Infrared Space Observatory, Thi *et al.*^{3,4} reported an abundance of H_2 in some of the same stars, as much as a mass equivalent to that of Jupiter. But this finding was subsequently challenged for one of the stars, β -Pictoris, by Lecavelier des Etangs *et al.*⁵ using data from the Far Ultraviolet Spectroscopic Explorer. More recent infrared observations by Richter *et al.*⁶ also seem to negate the claim of Thi *et al.*

Does this mean that Jupiter-like planets cannot form around those stars? Or have giant planets or protoplanets already formed⁵ in those systems, so that there is little H_2 left over in the disk to observe? Giant planets are clearly present in our Solar System and are also observed as extra-solar planets orbiting other stars. Are these giants exceptional? Questions abound.

Brittain and Rettig¹ introduce a new observational tool in this field — the infrared vibration–rotation spectrum of H_3^+ at a wavelength of 4 μm . The H_3^+ ion is an H_2 molecule with an extra proton attached. The third hydrogenic species (after H and H_2), it has only recently been introduced into astronomical observations, but its excellence as an astrophysical probe is rapidly being recognized⁷.

The H_3^+ spectrum has been seen in a wide variety of objects: in the giant planets

Jupiter⁸, Saturn and Uranus; in molecular clouds⁹; in the diffuse interstellar medium¹⁰; and possibly even in a supernova and an extragalactic object. The observations by Maillard *et al.*⁸ of intense H_3^+ emission in the auroral regions of Jupiter show a spectacularly pure spectrum: all the spectral lines seen are due to the H_3^+ ion and the spectrum is practically free of background radiation¹¹. The purity of the spectrum is such that it can be used to study plasma activities in the Jovian ionosphere from ground-based observatories simply by using an infrared camera with a proper filter.

So it seems natural to think of using the same spectrum to detect extra-solar giant planets, and indeed several projects are underway¹². The detection by Brittain and Rettig of H_3^+ in the protoplanetary disk of HD 141569 is therefore big news. Although HD 141569 is so far away (320 light years) compared with Jupiter (40 light minutes), the dilution of the signal with distance (by a factor of around 10^{-13}) might be compensated for by the larger amount of gaseous H_2 available in a gas-giant protoplanet. (In Jupiter most of the H_2 molecules are locked up in its interior as a high-density metallic fluid, so the planet has a high magnetic field¹¹.) Nevertheless, questions remain as to how H_2 gas is ionized and how H_3^+ ions are excited to the higher energy state of the emission.

How convincing is Brittain and Rettig's claim to have detected H_3^+ emission? Frequency matching between astronomical and laboratory spectra has been the cornerstone of identifying molecules in space. The discriminative power of high-resolution spectroscopy is so great that the discoveries of important molecules such as H_2O , H_2CO , CO and HC_3N in space were all claimed on the strength of the detection of just one spectral line, and confirmed later by the observation of other lines. In view of this, Brittain and Rettig's observation of two spectral lines (shown in Fig. 2a and Fig. 2b on page 58), at the right frequencies, seems more than sufficient. The latter line is perhaps less convincing, but the authors' subsequent observations have confirmed this line with a higher signal-to-noise ratio (T. Rettig, personal communication).

However, a few enigmas remain. In particular, Brittain and Rettig note that another H_3^+ line is missing from their spectrum. This is in stark contrast to the data from Jupiter, in which that line was observed with an intensity comparable to that of one of the lines that Brittain and Rettig do see. There are also a few other details in the data that I find difficult to explain — more observations are needed to firmly establish H_3^+ emission in HD 141569.

Brittain and Rettig's findings will certainly stimulate interest in the formation of giant planets and H_3^+ spectroscopy. No doubt many of the world's infrared telescopes will

be pointed towards HD 141569 and other young stars this year. It will be wonderful to see H_3^+ promoted as an essential probe for the studies of gas-giant protoplanets.

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1. Brittain, S. D. & Rettig, T. W. *Nature* **418**, 57–59 (2002).
2. Zuckerman, B., Forveille, T. & Kastner, J. H. *Nature*

373, 494–496 (1995).

3. Thi, W. F. *et al.* *Nature* **409**, 60–63 (2001)
4. Thi, W. F. *et al.* *Astrophys. J.* **561**, 1074–1094 (2001).
5. Lecavelier des Etangs, A. *et al.* *Nature* **412**, 706–708 (2001).
6. Richter, M. J., Jaffe, D. T., Blake, G. A. & Lacy, J. H. Preprint astro-ph/0205301 (2002); <http://xxx.lanl.gov>
7. McCall, B. J. & Oka, T. *Science* **287**, 1941–1942 (2000).
8. Maillard, J.-P., Drossart, P., Watson, J. K. G., Kim, S. J. & Caldwell, J. *Astrophys. J.* **363**, L37–L41 (1990).
9. Geballe, T. R. & Oka, T. *Nature* **384**, 334–335 (1996).
10. McCall, B. J., Geballe, T. R., Hinkle, K. H. & Oka, T. *Science* **279**, 1910–1913 (1998).
11. Oka, T. *Rev. Mod. Phys.* **64**, 1141–1149 (1992).
12. Miller, S. *et al.* *Phil. Trans. R. Soc. Lond. A* **358**, 2485–2502 (2000).

Metabolism

Demand management in cells

Stephen Oliver

Many researchers have tried to increase the flux through metabolic pathways in cells by raising the supply of metabolic enzymes. Little attention has been paid to the demand side of the equation — until now.

Metabolic engineering is a frustrating occupation. The aim is to genetically manipulate a cell — usually a microorganism — so that it overproduces some desired product. The trouble is that it is the business of microbes to produce more microbes, not to divert resources into the wholesale generation of a protein or other metabolite that might be desirable to the metabolic engineer but whose overproduction is of no advantage to the organism itself. So, in trying to increase the flow of intermediates through a metabolic pathway, the metabolic engineer is working against the complex controls of the cell, which act to ensure that this flux remains constant. Writing in *Journal of Bacteriology*, however, Koebmann and colleagues¹ describe how they tricked *Escherichia coli* bacteria into doing what the authors wanted.

Cellular controls on metabolism are subtle and difficult to analyse. Until recently, metabolic engineers have worked on the premise that there is a single rate-determining step in any metabolic pathway. Increasing the flux through a pathway should then be a simple business of increasing the active concentration of the enzyme catalysing that step. This is easy to achieve with recombinant DNA technology. The number of copies of the gene encoding the rate-determining enzyme can be increased — for instance, by cloning onto multiple DNA molecules (plasmids) for insertion into the cell. Alternatively, the expression of the gene can be set to a higher level by cloning it behind a high-efficiency gene-control region. But, while conceptually and technically simple, this strategy seldom works.

The problem is that the concept is wrong. Single enzymatic steps rarely determine the flux through a pathway; instead, control is shared between many of the pathway's

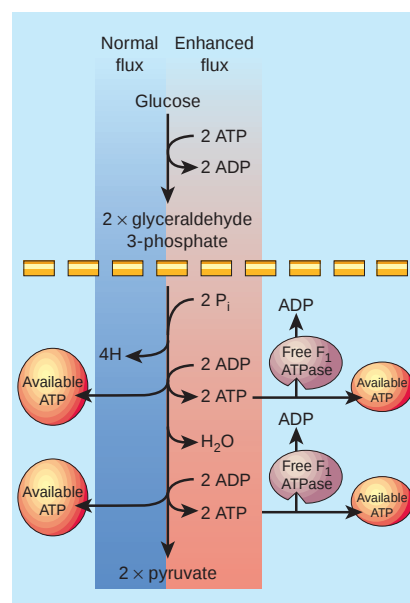


Figure 1 A simplified view of glycolysis. Above the dashed bar, the pathway consumes two molecules of ATP. Below, it generates four molecules of ATP — making a net gain of two ATP molecules available to supply energy for cellular processes. In the normal *E. coli* strain (left), all of the ATP generated is available to the cell. In the genetically engineered strain described by Koebmann *et al.*¹ (right), some of this ATP is degraded by the free F_1 -ATPase enzyme, making less available for use by the cell, but enhancing the flux through the glycolytic pathway.

enzymes. This is a truth that was first realized by Kacser² when he proposed his method of 'metabolic control analysis' nearly 30 years ago. The idea has been gaining adherents (often called 'controlniks') ever since, and started to be noticed by metabolic engineers

when Kacser, Niederberger and colleagues³ investigated the biosynthesis of the amino acid tryptophan in yeast. They found that a significant increase in flux, leading to the overproduction of tryptophan, required an increase in the copy number of all the genes encoding the necessary enzymes, not just one of them.

Even so, these enzymes were together responsible for only 26% of the control of flux through the pathway. So the manipulation of this pathway in yeast was a successful demonstration of the power of metabolic control analysis, but only a modest increase in the rate of synthesis of the amino acid was achieved. Kacser and colleagues had worked to increase the supply side of the equation. They did not, however, consider the demand for tryptophan — they never measured the flux due to the consumption of the amino acid in protein synthesis. What if, in the management of the cellular economy, it is demand, not supply, that dominates?

Koebmann *et al.*¹ have now tested this idea by looking at glycolysis — the basic pathway that derives useful energy, in the form of adenosine triphosphate (ATP), from the breakdown of glucose in all organisms. As with tryptophan biosynthesis, attempts to manipulate the control of glycolysis have looked at the supply side of the cell's economic equation. Various ways have been tried to speed up glycolysis by increasing the activity of the enzymes that degrade glucose, and they have all failed.

Instead, Koebmann *et al.* decided to increase the demand for ATP in *E. coli*. They achieved this by lowering the concentration of ATP in the bacterium through overproduction of the enzyme F_1 -ATPase. This enzyme is normally anchored to the bacterial cell membrane, where its role is to synthesize ATP in the respiratory pathway that follows on from glycolysis. But Koebmann *et al.* engineered *E. coli* to make F_1 -ATPase free from its membrane anchor. Now, instead of synthesizing ATP, the enzyme degrades it. From the cell's viewpoint, all of its energy-requiring metabolic reactions seem to have been stepped up — there appears to be an increased demand for ATP. And what the authors found was that the greater the activity of the ATP-degrading enzyme (that is, the higher the cell's demand for ATP), the greater was the flux through glycolysis. The cells also generated more of another end-product of glycolysis — pyruvate (or rather acetate, which is derived directly from it).

Demand management is a model of metabolism that is much loved by one group of controlniks⁴, and for which Koebmann *et al.*¹ have now provided compelling experimental evidence. The full story might be a little more complex because, as Fig. 1 shows, the first part of the glycolytic pathway creates its own demand for ATP, and it is unclear how enough ATP is channelled in this direc-

tion to sustain the increase in flux. Nevertheless, these results have implications for understanding the control of glycolysis in all organisms. They might enable us to brew beer faster and to generate more alcohol, and might also suggest new treatments for diseases, such as diabetes, in which the control of sugar metabolism goes wrong. Moreover, they could mean that biologists in the twenty-first century need a rethink of their view of cellular economy that is every bit as radical as that initiated for political economy

by John Stuart Mill and William Stanley Jevons in the nineteenth century. ■

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1. Koebmann, B. J., Westerhoff, H. V., Snoep, J. L., Nilsson, D. & Jensen, P. R. *J. Bacteriol.* **184**, 3909–3916 (2002).
2. Kacser, H. & Burns, J. A. *Symp. Soc. Exp. Biol.* **27**, 65–104 (1973).
3. Niederberger, P., Prasad, R., Miozzari, G. & Kacser, H. *Biochem. J.* **287**, 473–479 (1992).
4. Hofmeyr, J.-H. & Cornish-Bowden, A. *FEBS Lett.* **467**, 47–51 (2000).

Relativity

Special treatment

Giovanni Amelino-Camelia

The detection of cosmic rays with unexpectedly high energies has prompted a rethink of Einstein's theory of special relativity. A new formulation, called 'doubly special relativity', might be the answer.

At the turn of the twentieth century, the theory of space and time that had originated with Galileo and Newton was abandoned — it was incompatible with the mathematical structure of Maxwell's equations for electromagnetism and in conflict with the results of the Michelson–Morley experiments that had disproved the existence of the aether. Galileo–Newton theory was superseded by Einstein's theory of special relativity, but, after a century of success, that too is now being questioned. The observation of ultra-high-energy cosmic rays¹ seems to be in conflict with a key prediction

of special relativity, and some approaches to the construction of 'quantum gravity' — a theory that would unite quantum mechanics and general relativity — seem to require² the introduction of a new absolute scale in the theory of special relativity. Writing in *Physical Review Letters*, Magueijo and Smolin³ propose a scheme for the modification of special relativity, in which a new absolute scale sets simultaneously the maximum energy and maximum momentum of fundamental particles.

Special relativity has only one absolute scale: the speed of light is the same for all



Figure 1 Cosmic-ray shower. High-energy particles from distant galaxies impinge on the Earth's atmosphere, generating showers of secondary particles, including electrons, protons, muons and neutrinos. By detecting these secondary particles, the energy of the original cosmic ray can be reconstructed — but it seems that some cosmic rays have energies higher than is theoretically allowed¹. Modifying Einstein's theory of special relativity could account for this anomaly: Magueijo and Smolin³ present a new theory based on the idea² of 'doubly special relativity', in which the Planck energy scale is introduced as a second absolute scale, alongside the speed of light.

observers, in all frames of reference; for particles with mass, the speed of light is the maximum attainable velocity. Last year, I proposed² that the introduction of a second absolute scale, changing the postulates of special relativity, would fit the needs of some quantum-gravity approaches and would affect the analysis of cosmic rays. The corresponding relativistic theories are called 'doubly special', to emphasize the doubling of the number of absolute scales. For nearly a year, follow-up studies⁴ seemed to suggest that the only mathematically consistent doubly special relativity theory would be the one I had used² to illustrate the idea. Unfortunately that theory does not provide a satisfactory explanation for the observations of ultra-high-energy cosmic rays. Magueijo and Smolin³ have now succeeded in obtaining a second example of a doubly special theory. As well as the appealing feature of the second absolute scale as an energy–momentum maximum, the Magueijo–Smolin analysis might also lead to an explanation of the puzzling observations of ultra-high-energy cosmic rays.

The aspect of cosmic-ray observations that is most relevant for special relativity is known as the Greisen–Zatsepin–Kuzmin (GZK) limit. Cosmic rays are high-energy particles that are produced in distant galaxies and are detected on Earth through the particle-cascade processes that they ignite in the atmosphere (Fig. 1). But cosmic rays with energies above a certain threshold value — the GZK limit — should not reach Earth, interacting instead with the cosmic microwave background radiation (the relic radiation of the Big Bang). In the framework of special relativity, the GZK limit is around 5×10^{19} electron volts (eV), but several cosmic rays have been detected with energies higher than this¹. Of course, there is always the possibility that the experimental data have been misinterpreted, but, if the GZK description is valid, these observations encourage a rethink of special relativity.

There is also support for a rethink from the theoretical side. The energy/momentum scale known as the Planck scale, equivalent to 10^{28} eV, naturally has a special role in attempts to unify quantum mechanics and general relativity into quantum gravity⁵, as this scale is a combination of the quantum-mechanical Planck constant, the gravitational constant and the speed-of-light constant. Quantum gravity is likely to require^{2,3,5} a new quantum picture of space-time for particles with energies and momenta above the Planck scale, although for particles below the Planck scale the present classical picture would remain valid. But this poses a relativistic problem: special relativity predicts that the same particle should have energy higher than the Planck scale according to some observers, and energy lower than the Planck scale according to other observers. It would

be paradoxical if different observers disagreed on whether quantum space-time effects are present in a process. Doubly special relativity can solve² this puzzle. In particular, the theory proposed by Magueijo and Smolin³ predicts that all observers agree on whether or not a particle has energy and momentum smaller than the Planck scale.

The introduction of the Planck scale as an absolute relativistic scale also modifies the GZK limit. Just as Einstein's absolute velocity scale required a new law for combining velocities (so that the velocity of the source does not affect the velocity of light), the existence of an absolute energy/momentum scale would affect the laws for combining energies and momenta. In turn, these laws affect the GZK limit through energy–momentum conservation in the interactions between cosmic rays and the cosmic microwave background radiation. So Magueijo and Smolin's modification to introduce the energy/momentum scale means that the GZK limit is also modified. A similar mechanism is at work in the previous example of doubly special relativity I had constructed², but there the Planck scale only sets the maximum value of momentum (energies remain unbounded). Even the more pervasive role of the Planck scale in the Magueijo–Smolin theory might still be insufficient⁶ to resolve the cosmic-ray paradox fully. But the realization that the magnitude of the effects introduced by doubly special relativity depends

strongly on the way in which the second absolute scale is introduced has generated considerable interest^{7–9}. It is now reasonable to hope that a satisfactory relativistic explanation of the cosmic-ray paradox might be within reach.

More theoretical and experimental work is needed to establish whether another relativity revolution is really upon us. Doubly special relativity also affects the laws of particle propagation, and experiments on board the GLAST telescope, operational from 2006, will be sensitive¹⁰ to such effects. Then we will know whether doubly special relativity is just an amusing toy for theoretical physicists — or whether it should replace Einstein's theory of special relativity. ■

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1. Bird, D. J. *et al.* *Astrophys. J.* **441**, 144–150 (1995).
2. Amelino-Camelia, G. *Phys. Lett. B* **510**, 255–263 (2001).
3. Magueijo, J. & Smolin, L. *Phys. Rev. Lett.* **88**, 190403 (2002).
4. Kowalski-Glikman, J. *Mod. Phys. Lett. A* **17**, 1–12 (2002).
5. Carlip, S. *Rep. Prog. Phys.* **64**, 885–942 (2001).
6. Amelino-Camelia, G., Benedetti, D. & D'Andrea, F. Preprint hep-th/0201245 (2002); <http://xxx.lanl.gov>
7. Kowalski-Glikman, J. & Nowak, S. Preprint hep-th/0203040 (2002); <http://xxx.lanl.gov>
8. Lukierski, J. & Nowicki, A. Preprint hep-th/0203065 (2002); <http://xxx.lanl.gov>
9. Jüdes, S. & Visser, M. Preprint gr-qc/0205067 (2002); <http://xxx.lanl.gov>
10. Amelino-Camelia, G., Ellis, J., Mavromatos, N. E., Nanopoulos, D. V. & Sarkar, S. *Nature* **393**, 763–765 (1998).

Palaeontology

Early land vertebrates

Robert Carroll

A 350-million-year-old fossil provides evidence of an almost unknown stage in the origin of land vertebrates. It is also a reminder of how little is known of the relationships between the main lineages of amphibians and reptiles.

The transition between fish and land vertebrates was a turning point in the history of life. Early stages in the evolution from aquatic lobe-finned fish to tetrapods — animals with limbs capable of locomotion on land — are seen in many fossils from the Upper Devonian¹, just before 363 million years ago. In contrast, few remains are known from the next 30 million years, when the ancestors of the major tetrapod lineages differentiated from one another, an interval that falls in the early part of the Carboniferous period. So striking is the hiatus that Coates and Clack² coined the term 'Romer's Gap' for it, in reference to Alfred Sherwood Romer's long search for fossils dating to that time. It is fitting that Clack should be the first to recognize a well-preserved specimen of a new amphibian species from Romer's Gap. The fossil, *Pederpes finneyae*, comes from 350-million-year-old deposits at Dumbarton,

Scotland, and is described on page 72 of this issue³. The temporal and evolutionary context is shown in Fig. 1, overleaf.

Although the full length of the tail of *Pederpes* is not known, the animal was probably nearly a metre in length. It was a short-limbed, large-skulled predator, resembling an especially ungainly crocodile. But it almost certainly reproduced in the water, somewhat like modern aquatic salamanders. Grooves in the skull for lateral-line canals — a characteristic of fish — suggest that it lived partly in the water. The foot structure, however, indicates it could walk on land. *Pederpes* is advanced over its Devonian antecedents in having only five toes on the foot, yet has a relic of a tiny finger on the forelimb reminiscent of the supernumerary digits of the best-known amphibians — *Ichthyostega* and *Acanthostega* — from the Upper Devonian.

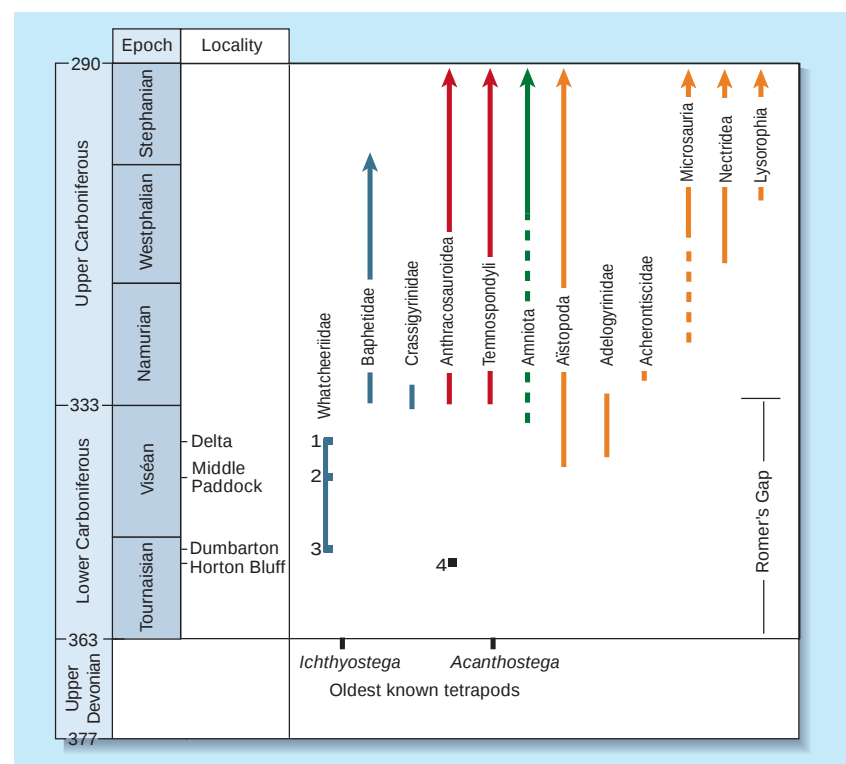


Figure 1 Temporal and evolutionary context for the newly described amphibian *Pederpes*³. This time scale shows the extent of Romer's Gap and the fossil record of the major lineages of tetrapods evident during the Carboniferous. Ages are in millions of years. Numbers 1–3 designate sites from which whatcheeriids (which include *Pederpes*) have been found: Delta Iowa, North America⁹; Middle Paddock, Australia¹⁰; and Dumbarton, Scotland⁵. The oldest site from which any Carboniferous tetrapods have been discovered is (4) Horton Bluff, Nova Scotia⁸. The main lineages shown here are as follows. Blue, the family Whatcheeriidae and two other lineages that are intermediate in their anatomy between Devonian amphibians and other early tetrapods. Red, the anthracosaurs and temnospondyls, 'conservative' amphibian lineages that were dominant in the later Palaeozoic and early in the ensuing Mesozoic era. Green, amniotes, the ancestors of modern reptiles, birds and mammals. Orange, the six forms of lepospondyls.

Discovery of a nearly complete skeleton in the middle of Romer's Gap should help in establishing the pattern of evolutionary change among early tetrapods. It might also provide context for understanding the inter-relationships of all later land vertebrates.

Clack shows that *Pederpes* and other members of the Whatcheeriidae — the family to which it is assigned — occupied an intermediate grade between the primarily aquatic Upper Devonian amphibians and later tetrapods. In particular, the feet show major advances towards effective locomotion on land. On the other hand, whatcheeriids have no skeletal features that indicate specific affinities to either of the major groups of 'conservative' amphibians, the temnospondyls or anthracosaurs, that dominated the later Carboniferous.

Even greater differences in skeletal anatomy and presumed ways of life separate these groups from a diversity of much smaller, but more specialized, animals superficially resembling snakes, salamanders and primitive lizards, that lived later in the Carboniferous⁴. These include six groups of

lepospondyls, all of which presumably retained a broadly amphibious way of life, and early representatives of the lineage that led to amniotes (the ultimate ancestors of modern reptiles, birds and mammals). The most specialized lepospondyl group, the aistopods⁵, which had more than 80 trunk vertebrae but completely lacked limbs, are known from only ten million years after *Pederpes*; and a possible antecedent of amniotes, *Casineria kiddi*⁶, appears only five million years after the earliest aistopod. It is not until late in the Viséan epoch, approximately 338 million years ago, that a truly diverse fauna of terrestrial vertebrates is known⁷. But three major lineages of lepospondyls — the elongate, short-limbed lysorophids, the secondarily aquatic nectrideans and the microsauria — appear only later. No representative of any of these lineages is known from the Tournaisian, the epoch that preceded the Viséan and lies at the beginning of the Carboniferous, although they might be expected to have existed given the evolutionary relationships proposed by Clack.

Perhaps the greatest importance of *Pederpes* is to emphasize the absence of fossils of so many other early tetrapod lineages that might be expected near the beginning of the Carboniferous. The only other Tournaisian site from which terrestrial vertebrates have been discovered is the slightly older Horton locality in eastern Canada, from which numerous but disarticulated elements of anthracosaurs have been found⁸.

Discoveries of fossils representing well-established groups, such as dinosaurs and Cenozoic mammals, occur comparatively frequently. But the number of truly intermediate forms linking the major groups of vertebrates remains small. Presumably this reflects the higher probability of finding fossils of long-lived and widely distributed groups, such as the whatcheeriids, which are known from Scotland, North America and Australia, and span 15 million years, a comparatively long time. In contrast, the many distinct but perhaps rare and short-lived lineages that linked ancestral and descendant forms across major evolutionary radiations remain largely unknown. Such radiations include those of early Palaeozoic fish and the diverse orders of placental mammals, as well as that of early land vertebrates.

The absence of fossils of early members of most Palaeozoic lineages also precludes the possibility of establishing a reliable classification of the main groups of living tetrapods. Without knowledge of the earliest recognizable ancestors of the groups leading to amniotes and the three orders of modern amphibians (frogs, salamanders and caecilians), we cannot determine whether the modern amphibians had an immediate common ancestry or which of the Palaeozoic lineages is the closest relative of amniotes. Clack's discovery raises hopes that further searches in Lower Carboniferous deposits, especially in Scotland, may finally provide an answer to these questions.

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1. Clack, J. A. in *Amphibian Biology* (eds Heatwole, H. & Carroll, R. L.) 979–1029 (Surrey Beatty, Chipping Norton, Australia, 2000).
2. Coates, M. I. & Clack, J. A. in *Studies on Early Vertebrates* (eds Arsenault, M., Lelièvre, H. & Janvier, P.) 373–388 (Bulletin du Muséum National d'Histoire Naturelle, Paris, 1995).
3. Clack, J. A. *Nature* **418**, 72–76 (2002).
4. Carroll, R. L. in *Amphibian Biology* (eds Heatwole, H. & Carroll, R. L.) 1198–1269 (Surrey Beatty, Chipping Norton, Australia, 2000).
5. Anderson, J. S. *Systematic Biol.* **50**, 170–193 (2001).
6. Paton, R. L., Smithson, T. R. & Clack, J. A. *Nature* **398**, 508–513 (1999).
7. Rolfe, W. D. I., Clarkson, E. N. K. & Panchen, A. L. (eds) *Trans. R. Soc. Edinburgh: Earth Sci.* **84**, 175–464 (1994).
8. Clack, J. A. & Carroll, R. L. in *Amphibian Biology* (eds Heatwole, H. & Carroll, R. L.) 1030–1043 (Surrey Beatty, Chipping Norton, Australia, 2000).
9. Bolt, J. R. & Lombard, R. E. in *Amphibian Biology* (eds Heatwole, H. & Carroll, R. L.) 1044–1052 (Surrey Beatty, Chipping Norton, Australia, 2000).
10. Thulborn, T., Warren, A., Turner, S. & Hamley, T. *Nature* **381**, 777–780 (1996).

Conserved function for embryonic nodal cilia

A similar mechanism may underlie the handedness seen in all vertebrate body plans.

How left–right handedness originates in the body plan of the developing vertebrate embryo is a subject of considerable debate^{1,2}. In mice, a left–right bias is thought to arise from a directional extracellular flow (nodal flow) that is generated by dynein-dependent rotation of monocilia on the ventral surface of the embryonic node^{3,4}. Here we show that the existence of node monocilia and the expression of a dynein gene that is implicated in ciliary function are conserved across a wide range of vertebrate classes, indicating that a similar ciliary mechanism may underlie the establishment of handedness in all vertebrates.

In mice, mutations in the gene that encodes the left–right dynein heavy chain (*Lrd*), a component of the ciliary motor, result in immotile node cilia and a reversal of the left–right (L–R) axis in roughly half of mutant offspring^{5,6}. In humans, mutations in the dynein heavy-chain gene *DNAH5* are also associated with immotile cilia syndrome, an inherited disorder that includes mirror-image reversal of the internal organs in half of affected individuals⁷. Both *Lrd* (refs 5, 6) and *Dnahc5* (ref. 7) are expressed in the ventral layer of the mouse node in cells containing cilia (Fig. 1a–c).

To test the validity and universality of the nodal-flow model, we cloned *Lrd* homologues from chick, *Xenopus* and zebrafish, analysed their expression, and examined embryos for the presence of monocilia (see supplementary information). In chick embryos, *Lrd* (*Lrd*-related) is expressed during gastrulation within Hensen's node and in the primitive streak (Fig. 1d). Although it has been suggested that there is no equivalent of the ventral mouse node in chick⁸, we observed cilia at Hensen's node projecting ventrally from the epiblast layer into the space between the dorsal epiblast and the ventral endoderm (Fig. 1e, f).

In *Xenopus*, *Lrd* is expressed at the end of gastrulation on the ventral side of the dorsal blastopore (Fig. 1g), in cells derived from the early gastrula organizer. At the same time, cilia arise in this region at the completion of gastrulation (Fig. 1h, i). Similarly, a zebrafish *Lrd* is expressed at the end of gastrulation in the dorsal forerunner cells that migrate ahead of the organizer region (Fig. 1j). Unique to fish, these dorsal forerunner cells give rise during early somite stages to Kupffer's vesicle, a structure that is found in the tail region ventral to the chordal–neural hinge⁹. Strikingly, we found that cells within Kupffer's vesicle also contain monocilia (Fig. 1k, l).

The conservation of nodal cilia and the

conserved expression of *Lrd* homologues in different vertebrate model organisms indicate that the activity of nodal cilia is probably a universal mechanism for specifying

the vertebrate L–R axis. Previous failures to identify nodal cilia in non-mammals may be related to the differences we have observed in the relative timing and

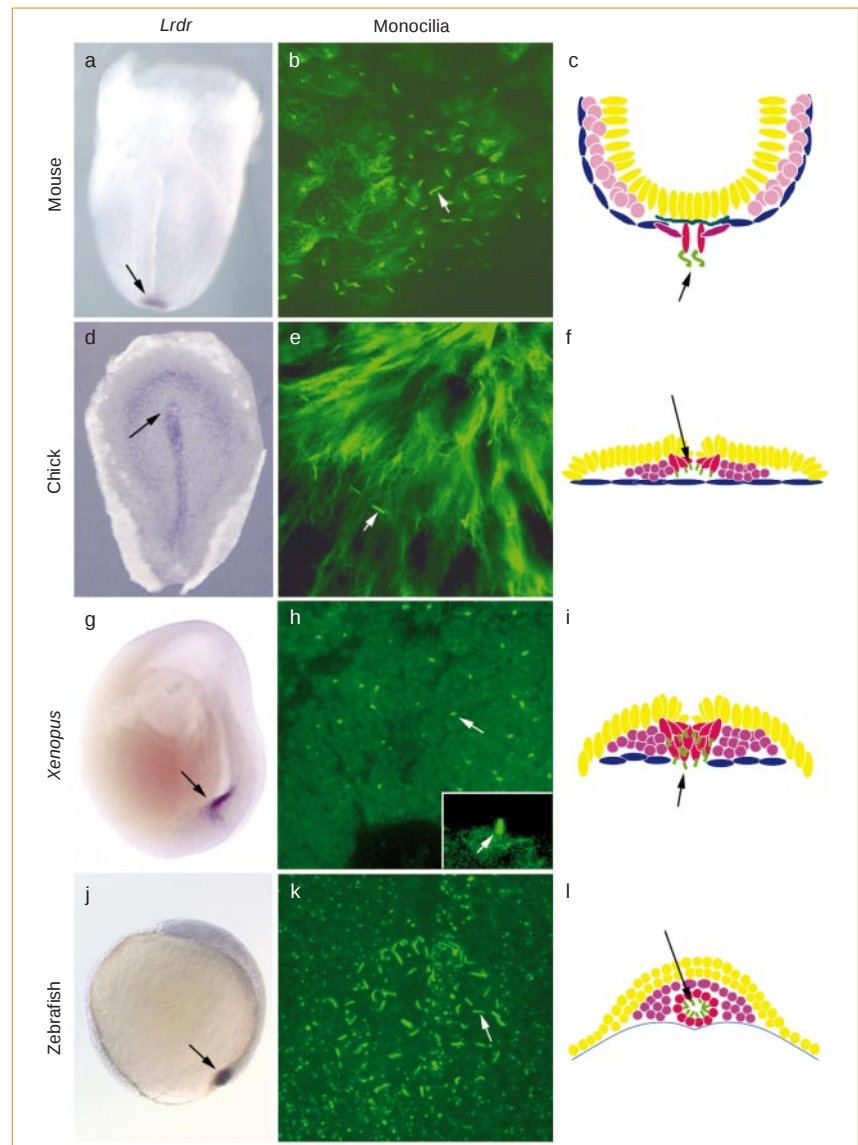


Figure 1 Regions of potential left–right signalling in mouse, chick, *Xenopus* and zebrafish. These regions were identified by *in situ* hybridization with *Lrd* probes (left) and immunofluorescence with an antibody against acetylated tubulin to detect cilia (centre). Drawings (right) show the locations of ciliated cells. **a**, Frontal view of a mouse embryo 7.5 days post-fertilization (d.p.f.), showing *Lrd* expression at the node. **b**, Ventral view of a mouse embryo at 7.5 d.p.f., showing cilia on the ventral surface of the node. **d**, Dorsal view of a Hamburger–Hamilton (HH) stage 4— chick gastrula-stage embryo showing *Lrd* expression in Hensen's node and in the primitive streak. **e**, In chick, cilia are present at Hensen's node at HH stage 4— in the space between the dorsal epiblast and the ventral endoderm. **g**, Lateral view of a stage-13 *Xenopus* embryo, showing *Lrd* expression in the ventral cells of the dorsal blastopore. **h**, After the completion of gastrulation in *Xenopus* (stage 14), cilia begin to form on the ventral surface of the dorsal blastopore. Inset, lateral view of a single cilium. **j**, Lateral view of a late gastrula, or 90% epiboly, zebrafish embryo, showing expression in the dorsal forerunner cells ahead of the organizer region. **k**, In zebrafish at the 10-somite stage (dorsal view), cells in Kupffer's vesicle are ciliated on their inner surface. Anterior is towards the top in all panels; black arrows show regions of *Lrd* expression where cilia are found (or subsequently develop); white arrows show cilia. **c**, **f**, **i**, **l**, Transverse sections (dorsal is towards the top) showing the locations of ciliated cells (red/green) with respect to the mesoderm (pink), ectoderm (yellow) and endoderm (purple). Arrows indicate the views used in central panels.

Table 1 Nodal cilia features in different vertebrate classes

	Onset of <i>Lrd</i> mRNA expression	Appearance of nodal cilia	Earliest conserved asymmetric gene expression
Mouse	Gastrula, 7.5 d.p.f.	Gastrula, 7.5 d.p.f.	<i>Nodal</i> , 8.25 d.p.f., 3–5 somites
Chick	Gastrula, HH4 –	Gastrula, HH4 –	<i>Nodal</i> , HH7, 0–2 somites
<i>Xenopus</i>	Stage-11 gastrula	Stage-14 neurula	<i>Xnr-1 (Nodal)</i> , stage-17 neurula
Zebrafish	80% epiboly gastrula	Four somites	<i>Cyclops (Nodal)</i> , 20 somites

*Asymmetric gene expression of *Sonic hedgehog* at the node occurs at HH5 (gastrulation) before *Nodal* expression, but seems to be unique to the chick embryo. d.p.f., days post-fertilization.

localization of *Lrd* expression and formation of nodal cilia. The earliest known asymmetric expression patterns that are common to all vertebrates likewise exhibit considerable variability in their time of onset among different vertebrate classes^{1,2}. In all instances, however, these conserved asymmetries are preceded by the onset of *Lrd* expression and by the appearance of nodal cilia (Table 1), indicating that nodal cilia may be responsible for initiating L–R asymmetric gene expression and for establishing the final body plan in all vertebrates.

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1. Capdevila, J. et al. *Cell* **101**, 9–21 (2000).
2. Wright, C. V. E. *Dev. Cell* **1**, 179–186 (2001).
3. Nonaka, S. et al. *Cell* **95**, 829–837 (1998).
4. Okada, Y. et al. *Mol. Cell* **4**, 459–468 (1999).
5. Supp, D. M. et al. *Nature* **389**, 963–966 (1997).
6. Supp, D. M. et al. *Development* **126**, 5495–5504 (1999).
7. Olbrich, H. et al. *Nature Genet.* **30**, 143–144 (2002).
8. Männer, J. *Anat. Embryol.* **203**, 481–490 (2001).
9. Cooper, M. S. & D'Amico, L. A. *Dev. Biol.* **180**, 184–198 (1996).

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Gene expression

RNA interference in adult mice

RNA interference is an evolutionarily conserved surveillance mechanism that responds to double-stranded RNA by sequence-specific silencing of homologous genes. Here we show that transgene expression can be suppressed in adult mice by synthetic small interfering RNAs and by small-hairpin RNAs transcribed *in vivo*

from DNA templates. We also show the therapeutic potential of this technique by demonstrating effective targeting of a sequence from hepatitis C virus by RNA interference *in vivo*.

Small interfering RNAs (siRNAs) mimic intermediates in the RNA-interference (RNAi) pathway and can silence genes in somatic cells without activating non-specific suppression by double-stranded RNA-dependent protein kinase¹. To investigate whether siRNAs also inhibit gene expression *in vivo*, we used a modification

of hydrodynamic transfection methods^{2–4} to deliver naked siRNAs to the livers of adult mice. Either an siRNA derived from firefly luciferase or an unrelated siRNA was co-injected with a luciferase-expression plasmid (for construct description and sequences, see supplementary information). We monitored luciferase expression in living animals using quantitative whole-body imaging⁵ (Fig. 1a, c), and found that it was dependent on reporter-plasmid dose (results not shown).

In each experiment, serum measurements of a co-injected human α -1 antitrypsin (hAAT) plasmid⁶ served to normalize transfection efficiency and to monitor non-specific translational inhibition. Average serum concentrations of hAAT after 74 h were similar in all groups.

Our results indicate that there was specific, siRNA-mediated inhibition of luciferase expression in adult mice ($P < 0.0115$) and that unrelated siRNAs had no effect ($P < 0.864$; Fig. 1a, b). In 11 independent experiments, luciferase siRNAs reduced luciferase expression (as judged by emitted light) by an average of 81% ($\pm 2.2\%$). These findings indicate that RNAi can downregulate gene expression in adult mice.

As RNAi degrades respiratory syncytial virus RNAs in culture⁷, we investigated whether RNAi could be directed against a human pathogenic RNA expressed in a mouse, namely that of hepatitis C virus (HCV). (Infection by HCV, an RNA virus that infects 1 in 40 people worldwide, is the most common reason for liver transplantation in the United States and Europe.) We fused the NS5B region (non-structural protein 5B, viral-polymerase-encoding region) of this virus with luciferase RNA and monitored RNAi by co-transfection *in vivo*. An

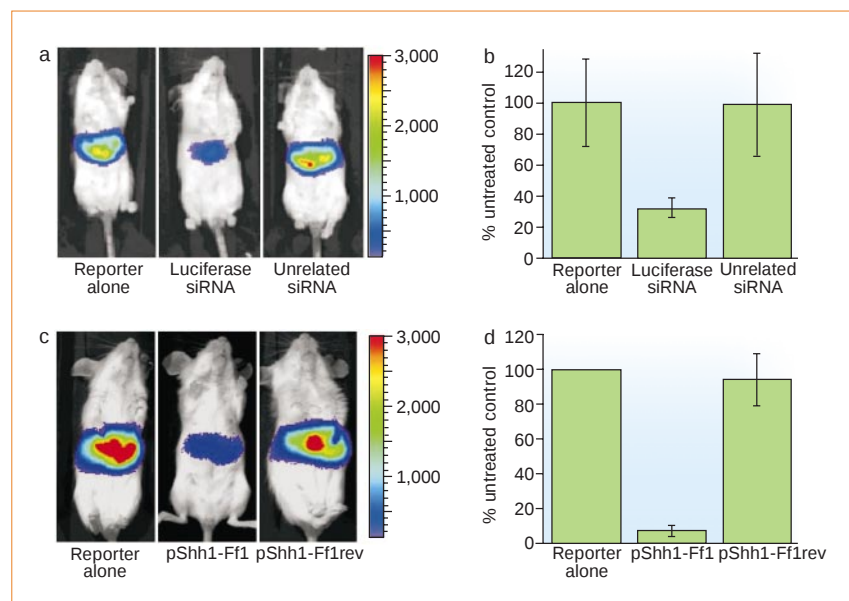


Figure 1 RNA interference in adult mice. **a**, Representative images of light emitted from mice co-transfected with the luciferase plasmid pGL3-control and either no siRNA, luciferase siRNA or unrelated siRNA. A pseudocolour image representing intensity of emitted light (red, most intense; blue, least intense) superimposed on a greyscale reference image (for orientation) shows that RNAi functions in adult mice. Annealed 21-nucleotide siRNAs (40 μ g; Dharmacon) were co-injected into the livers of mice with 2 μ g pGL3-control DNA (Promega) and 800 units of RNasin (Promega) in 1.8 ml PBS buffer in 5–7 s. After 72 h, mice were anaesthetized and given 3 mg luciferin intraperitoneally 15 min before imaging. **b**, siRNA results (six mice per group) from a representative experiment. Mice receiving luciferase siRNA emitted significantly less light than reporter-alone controls (one-way ANOVA with post hoc Fisher's test). Results for reporter alone and unrelated siRNA were statistically similar. **c**, pShh1-Ff1, but not pShh1-Ff1rev (see text), reduced luciferase expression in mice relative to the reporter-alone control. pShh1-Ff1 or pShh1-rev (10 μ g) were co-injected with 2 μ g pGL3-control in 1.8 ml PBS buffer. **d**, Average of three independent shRNA experiments ($n = 5$). Average values for the reporter-alone group are designated as 100% in each of the three experiments. Animals were treated according to the US National Institutes of Health's guidelines for animal care and the guidelines of Stanford University.

siRNA targeting the NS5B region reduced luciferase expression from the chimeric HCV NS5B protein–luciferase fusion by 75% ($\pm 6.8\%$; 6 animals per group). This result suggests that it may be feasible to use RNAi as a therapy against other important human pathogens.

Although our results show that siRNAs are functional in mice, delivery remains a major obstacle. Unlike siRNAs, functional small-hairpin RNAs (shRNAs) can be expressed *in vivo* from DNA templates using RNA polymerase III promoters^{8,9}; they are as effective as siRNAs in inducing gene suppression. Expression of a cognate shRNA (pShh1-Ff1; see supplementary information) inhibited luciferase expression by up to 98% ($\pm 0.6\%$), with an average suppression of 92.8% ($\pm 3.39\%$) in three independent experiments (Fig. 1c, d). An empty shRNA-expression vector had no effect (results not shown); reversing the orientation of the shRNA (pShh1-Ff1rev) insert prevents gene silencing because it alters the termination by RNA polymerase III and generates an improperly structured shRNA. These findings indicate that plasmid-encoded shRNAs can induce a potent and specific RNAi response in adult mice.

RNAi may find application in functional genomics or in identifying targets for designer drugs. It is a more promising system than gene-knockout mice because groups of genes can be simultaneously rendered ineffective without the need for time-consuming crosses. Gene therapy currently depends on the ectopic expression of exogenous proteins; however, RNAi may eventually complement this gain-of-function approach by silencing disease-related genes with DNA constructs that direct the expression of shRNAs. Our method of RNAi delivery could also be tailored to take advantage of developing viral and non-viral gene-transfer vectors in a clinical context.

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1. Elbashir, S. M. *et al.* *Nature* **411**, 494–498 (2001).
2. Zhang, G., Budker, V. & Wolff, J. A. *Hum. Gene Therapy* **10**, 1735–1737 (1999).
3. Liu, F., Song, Y. & Liu, D. *Gene Therapy* **6**, 1258–1266 (1999).
4. Chang, J., Sigal, L. J., Lerro, A. & Taylor, J. J. *Virol.* **75**, 3469–3473 (2001).
5. Contag, C. H. *et al.* *Photochem. Photobiol.* **66**, 523–531 (1997).
6. Yant, S. R. *et al.* *Nature Genet.* **25**, 35–41 (2000).
7. Bitko, V. & Barik, S. *BMC Microbiol.* **1**, 34 (2001).
8. Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J. & Conklin, D. S. *Genes Dev.* **16**, 948–958 (2002).
9. Tuschl, T. *Nature Biotechnol.* **20**, 446–448 (2002).

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COMMUNICATIONS ARISING

Orbital physics

Experimental quest for orbital waves

One challenge in condensed-matter physics is the experimental confirmation of a new kind of elementary excitation — orbital waves, or orbitons, which are predicted to exist in an orbitally ordered state. Saitoh *et al.*¹ have observed three peaks at 160, 144 and 126 meV in the Raman scattering of orbitally ordered lanthanum manganate (LaMnO₃), and interpret these as evidence of orbitons. However, we find similar peaks in the optical conductivity, $\sigma(\omega)$, of LaMnO₃ and point out that the direct observation of orbitons in $\sigma(\omega)$ is prohibited by a selection rule. This suggests that the Raman peaks observed by Saitoh *et al.* arise from multiphonons, and that the existence of orbitons has yet to be experimentally confirmed.

We determined $\sigma(\omega)$ by measuring both the transmittance and reflectance of single crystals, using a sample polished to a thickness of $d \approx 62 \mu\text{m}$ for the former. We com-

pared $\sigma(\omega)$ of LaMnO₃ with the Raman data of Saitoh *et al.*¹ and found that the peaks in $\sigma(\omega)$ were similar to the Raman features (Fig. 1, top), albeit with slightly different frequencies (160, 146, 130 and 118 meV).

The orbital excitations discussed by Saitoh *et al.* involve transitions between orbital states of the same parity. These transitions do not contribute directly to $\sigma(\omega)$ owing to the parity selection rule; that is, they are not infrared-active. They may become weakly infrared-active in the presence of defects or by the simultaneous excitation of a parity-breaking Mn–O bond-stretching phonon². The latter, phonon-activated mechanism is the more effective way to break the selection rule. Stronger features are therefore expected in $\sigma(\omega)$ at frequencies that — compared to the Raman peaks — are shifted by the respective phonon frequency of about 70 meV. As this is not the case, the peaks in $\sigma(\omega)$ cannot be explained by orbitons, challenging the orbiton interpretation of the Raman data.

We suggest that all peaks should be interpreted as arising from multiphonons. The sharp increase in $\sigma(\omega)$ at low frequen-

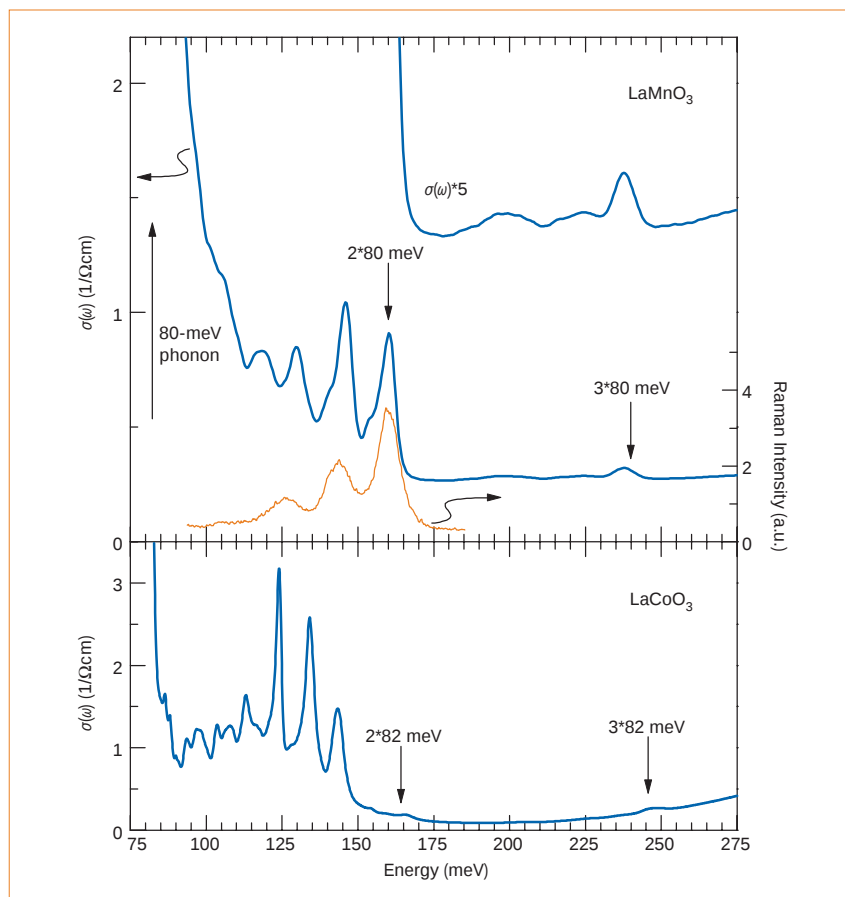


Figure 1 Optical conductivity $\sigma(\omega)$ of LaMnO₃ and LaCoO₃, and Raman measurements on LaMnO₃ at a temperature of 4 K. Top, comparison of $\sigma(\omega)$ (thick blue line) and Raman intensity (from Saitoh *et al.*¹; thin yellow line) of LaMnO₃ reveals a close similarity between the two spectra. In $\sigma(\omega)$ the highest peaks of single-, two- and three-phonon absorption are observed at 80, 160 and 240 meV, respectively. Inset, enlargement of the three-phonon range of $\sigma(\omega)$. Bottom, $\sigma(\omega)$ of LaCoO₃, showing multiphonon peaks, for example at 2*82 and 3*82 meV.

cies indicates the upper limit of (single) phonon absorption. The highest infrared-active phonon peak is observed at 80 meV (ref. 3), showing that the peaks at 160 and 240 meV are within the range of two- and three-phonon absorption, respectively. By increasing the number of phonons involved in the absorption process from one to two to three, the spectral weight in $\sigma(\omega)$ is reduced each time by one to two orders of magnitude. Moreover, multiphonon Raman scattering is predicted⁴ to be particularly strong in orbitally ordered LaMnO₃.

The multiphonon features do not necessarily correspond to simple multiples of the $k=0$ single-phonon peaks observed in Raman or $\sigma(\omega)$, because phonon modes from the entire Brillouin zone can be combined to yield the required total momentum, $k_{\text{tot}}=0$. The multiphonon peaks reflect peaks in the combined density of states weighted for each technique by the respective matrix element. In LaMnO₃, Raman and infrared spectroscopy are sensitive to excitations of different symmetry, which explains the slightly different peak frequencies observed using the two methods.

Below about 160 meV, multiphonon features are common in transition-metal oxides that have pseudocubic, perovskite structures. As an example, Fig. 1 shows $\sigma(\omega)$ for LaCoO₃, for which we measured the transmittance on a single crystal with $d \approx 37 \mu\text{m}$. At a temperature of 4 K, LaCoO₃ is in a non-magnetic state ($t_{2g}^6 e_g^0$) without orbital degeneracy or orbital order, and so orbital waves can be excluded as the origin of the observed peaks. These features must also be interpreted as arising from multiphonons.

Finally, we note that the peak frequencies are rather low for orbitons. Two components contribute to the orbiton energy: one is purely electronic and the other is due to electron–phonon coupling. Saitoh *et al.*¹ predict on theoretical grounds that the electronic contribution to the highest peak should be about $4J_1 \approx 200$ meV, and assume that the electron–phonon contribution is rather small ($0.56J_1 \approx 28$ meV). However, other estimates of the electron–phonon contribution^{5–11} vary between 0.7 and 2.0 eV and therefore do not support the existence of orbitons at 160 meV.

We conclude that the observed features of $\sigma(\omega)$ are due to multiphonons, and see no evidence so far to support an alternative explanation of the similar Raman peaks. Raman measurements on an oxygen-isotope-substituted sample of LaMnO₃ should clarify this point.

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1. Saitoh, E. *et al.* *Nature* **410**, 180–183 (2001).
2. Ballhausen, C. J. *Introduction to Ligand Field Theory* (McGraw-Hill, New York, 1962).
3. Paolone, A. *et al.* *Phys. Rev. B* **61**, 11255–11258 (2000).
4. Perebeinos, V. & Allen, P. B. *Phys. Rev. B* **64**, 085118 (2001).
5. Millis, A. J. *Phys. Rev. B* **53**, 8434–8441 (1996).
6. Pickett, W. E. & Singh, D. J. *Phys. Rev. B* **53**, 1146–1160 (1996).
7. Solovyev, I., Hamada, N. & Terakura, K. *Phys. Rev. B* **53**, 7158–7170 (1996).
8. Elfimov, I. S., Anisimov, V. I. & Sawatzky, G. A. *Phys. Rev. Lett.* **82**, 4264–4267 (1999).
9. Allen, P. B. & Perebeinos, V. *Phys. Rev. Lett.* **83**, 4828–4831 (1999).
10. Ahn, K. H. & Millis, A. J. *Phys. Rev. B* **61**, 13545–13559 (2000).
11. Bala, J. & Oles, A. M. *Phys. Rev. B* **62**, R6085–R6088 (2000).

Saitoh et al. reply — We disagree with the assertion of Grüninger *et al.* that the Raman peaks from LaMnO₃, which we interpret as being due to scattering from orbital excitation, are caused by multiphonon excitation.

First, the estimated oscillator strength, f , of their observed 160-meV peak in the optical conductivity, $\sigma(\omega)$, is less than 1×10^{-6} , which is much weaker than those of the main Mn–O stretching mode at 71 meV ($f \approx 2 \times 10^{-4}$) and of the electronic charge excitation at around 1.9 eV ($f \approx 2.7 \times 10^{-1}$; ref. 1). Such a weak infrared peak is explained by a spin-allowed $d-d$ electronic excitation that becomes infrared-active through the disruption of local-inversion symmetry due to impurities and/or defects. The orbiton represents one such low-energy $d-d$ excitation, although it can show k -dispersion.

Figure 1 shows the polarization dependence of the $\sigma(\omega)$ and Raman spectra for a detwinned LaMnO₃ crystal at about 10 K. As the $\sigma(\omega)$ was deduced from the reflectivity, the weak intensity (around $1 \Omega^{-1} \text{cm}^{-1}$) of the infrared-active peak is barely detectable. The $\sigma(\omega)$ spectra are also plotted

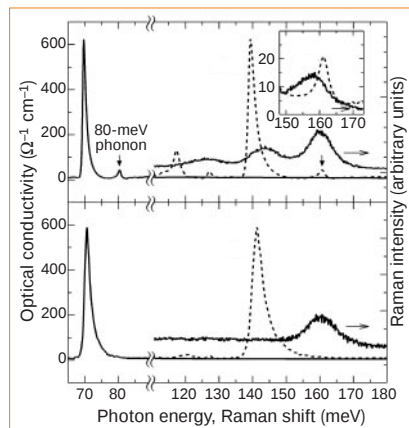


Figure 1 Polarized optical conductivity $\sigma(\omega)$ and Raman spectra in a detwinned LaMnO₃ crystal at around 10 K (inset, Raman spectrum at 300 K). Top, $E_{\perp z}$ spectra, (x,x) configuration; bottom, $E_{\parallel z}$ spectra, (z,z) configuration. The $\sigma(\omega)$ spectra are also plotted as functions of the doubled photon energy (dashed lines).

as functions of the doubled photon energy (Fig. 1, dashed lines). The infrared-active phonon at 80 meV is observed only in the $E_{\perp z}$ polarization ((x,x) configuration; Fig. 1, top). The $E_{\parallel z}$ spectra do not show any peak at the corresponding energy, although the prominent Raman band at 160 meV is evident in the (z,z) configuration (Fig. 1, bottom).

Any combination of two infrared-active modes with the same symmetry may produce the Raman-active symmetry. However, it is hard to reconcile a two-phonon interpretation in terms of an 80-meV in-plane-active mode, with the strong Raman intensity observed in the (z,z) configuration. In addition, the peak energy of the Raman band in the (x,x) configuration deviates from the doubled energy of the 80-meV phonon peak with increasing temperature (Fig. 1, inset).

In LaMnO₃, the 80-meV infrared-active mode in the $E_{\perp z}$ spectrum disappears above $T_J \approx 750$ K. In LaCoO₃, the $\sigma(\omega)$ spectra deduced from the reflectivity data do not show any prominent peak near 82 meV (ref. 2), in contrast to the 80-meV mode in LaMnO₃, which is weak but clearly discerned as the Jahn–Teller (JT) distortion-induced mode ($f \approx 1 \times 10^{-5}$). The origin of the 82-meV peak in LaCoO₃ observed by Grüninger *et al.* is interesting, but irrelevant to the origin of the intense, high-frequency Raman modes observed for LaMnO₃.

By considering both the electronic and phononic contributions to the orbiton energy equally, we have quantitatively estimated the parameter values of the interactions³. Using these values, we have calculated the Raman spectra that show good agreement with the experimental results. The large discrepancy between our and previous estimates, as pointed out by Grüninger *et al.*, arises from the absence or averaged treatment of the strong electron correlation.

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1. Tobe, K., Kimura, T., Okimoto, Y. & Tokura, Y. *Phys. Rev. B* **64**, 184421 (2001).
2. Yamaguchi, S., Okimoto, Y. & Tokura, Y. *Phys. Rev. B* **55**, R8666–R8669 (1997).
3. Okamoto, S., Ishihara, S. & Maekawa, S. *Phys. Rev. B* **65**, 144403 (2002).

Pluripotency of mesenchymal stem cells derived from adult marrow

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We report here that cells co-purifying with mesenchymal stem cells—termed here multipotent adult progenitor cells or MAPCs—differentiate, at the single cell level, not only into mesenchymal cells, but also cells with visceral mesoderm, neuroectoderm and endoderm characteristics *in vitro*. When injected into an early blastocyst, single MAPCs contribute to most, if not all, somatic cell types. On transplantation into a non-irradiated host, MAPCs engraft and differentiate to the haematopoietic lineage, in addition to the epithelium of liver, lung and gut. Engraftment in the haematopoietic system as well as the gastrointestinal tract is increased when MAPCs are transplanted in a minimally irradiated host. As MAPCs proliferate extensively without obvious senescence or loss of differentiation potential, they may be an ideal cell source for therapy of inherited or degenerative diseases.

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of the blastocyst that can be propagated indefinitely in an undifferentiated state. ES cells differentiate to all cell lineages *in vivo* and differentiate into many cell types *in vitro*. Although ES cells have been isolated from humans¹, their use in research as well as therapeutics is encumbered by ethical considerations². Stem cells also exist for most tissues, including haematopoietic³, neural⁴, gastrointestinal⁵, epidermal⁶, hepatic⁷ and mesenchymal stem cells⁸. Compared with ES cells, tissue-specific stem cells have less self-renewal ability and, although they differentiate into multiple lineages, they are not pluripotent.

Until recently, it was thought that tissue-specific stem cells could only differentiate into cells of the tissue of origin; however, recent studies suggested that tissue-specific stem cells can differentiate into lineages other than the tissue of origin. After transplantation of bone marrow or enriched haematopoietic stem cells (HSC), skeletal myoblasts^{9,10}, cardiac myoblasts^{11,12}, endothelium^{11–14}, hepatic and biliary duct epithelium^{15–17}, lung, gut and skin epithelia¹⁸, and neuroectodermal cells of donor origin have been detected^{19–22}. Some but not other studies demonstrated that neural stem cells^{23,24} as well as muscle cells^{25,26} may differentiate into haematopoietic cells. When injected into a blastocyst, neural stem cells contribute to a number of tissues of the chimaeric mouse embryo²⁷; however, most studies did not conclusively demonstrate that a single tissue-specific stem cell differentiates into functional cells of multiple tissues.

We identified a rare cell within human bone marrow mesenchymal stem cell cultures that can be expanded for more than 80 population doublings. This cell differentiates not only into mesenchymal lineage cells but also endothelium^{28,29} and endoderm³⁰. We show that cells capable of differentiating *in vitro* to cells of the three germ layers can be selected from rodent bone marrow. These cells contribute to most somatic tissues when injected into an early blastocyst and engraft *in vivo*, where they differentiate into tissue-specific cell types in response to cues provided by different organs.

Culture of undifferentiated mouse and rat MAPCs

To isolate MAPCs from murine bone marrow, we used methods identical to those used for human (h)MAPCs²⁸, except that murine

(m)MAPCs, but not hMAPCs, required leukaemia inhibitory factor (LIF) for expansion. (A detailed description of the culture method can be found in Supplementary Information Table 1.) We have been able to culture several mMAPC populations for more than 120 population doublings (Fig. 1a). The phenotype of mMAPCs in fresh bone marrow is unknown. The phenotype of cultured mMAPCs is CD34, CD44, CD45, c-Kit, and major histocompatibility complex (MHC) class I and II negative; mMAPCs express low levels of Flk-1, Sca-1 and Thy-1, and higher levels of CD13 and stage-specific antigen 1 (SSEA-1)¹ (Fig. 1b). The morphology and phenotype were similar after 30 to more than 120 population doublings (Supplementary Information Fig. 1). The mMAPC phenotype is similar to that of hMAPC²⁸ but different from that of murine haematopoietic stem cells with 'transdifferentiation' potential^{11,17,18,25}. mMAPCs were 8–10 μ m in diameter with a large nucleus and scant cytoplasm (Supplementary Information Fig. 1a). Average telomere length (ATL) of mMAPCs cultured for 40 population doublings was 27 kilobases (kb); when re-tested after 102 population doublings, ATL remained unchanged (Fig. 1c).

Similar results were obtained when we isolated and cultured MAPCs from bone marrow of Sprague–Dawley rats ($n = 3$). We have expanded rat (r)MAPCs for more than 100 population doublings (Supplementary Information Fig. 2a). Successful culture of rMAPCs required addition of LIF to epidermal growth factor (EGF) and platelet-derived growth factor (PDGF)-BB. rMAPCs were MHC class I and class II negative, CD44 negative (data not shown), expressed high levels of telomerase, and the telomeres have not shortened in culture for 100 population doublings (Supplementary Information Fig. 2b).

The finding that rodent but not hMAPCs require addition of LIF to the culture medium is similar to results for ES cells: human ES cells seem to be LIF-independent³¹ whereas murine ES cells require LIF for *ex vivo* maintenance³². Quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) showed that two transcription factors important in maintaining undifferentiated ES cells, Oct-4 (ref. 33) and Rex-1 (ref. 34), were expressed in mMAPCs: Rex-1 at levels similar to mouse ES cells and Oct-4 at levels 1,000-fold lower than ES cells (Fig. 1d).

In vitro differentiation of single mMAPCs and rMAPCs

Approximately 1% of wells seeded with ten CD45⁺ TER119⁺ bone marrow monoclonal cells (BMMNCs) yielded continuous growing cultures, suggesting that 1 out of 1,000 CD45⁺ TER119⁺ BMMNCs is capable of initiating MAPC cultures, and that progeny generated from ten cells is probably derived from one cell. To definitively prove that a single cell gives rise to continuous growing cultures and differentiated progeny, we used retroviral marking (Fig. 2; see also Supplementary Information Table 2). After sub-cloning at ten cells per well, three murine and two rat enhanced green fluorescent protein-expressing (eGFP⁺) cell populations were selected and culture-expanded for more than 100 population doublings. One hundred per cent of cells continued to express GFP after expansion. Southern blot analyses showed that a single retroviral insert was

detected in one mMAPC and one rMAPC population derived from ten initial cells (Fig. 2). The same single retroviral insert was also seen in seven subclones of the rMAPC population, demonstrating that progeny of only a single eGFP-transduced mMAPC or rMAPC gave rise to the continuous growing populations^{35,36}. We sequenced the genomic flanking region 3' from the retrovirus using splinkerette PCR, which detects a single retroviral insert in as few as 5 × 10³ cells³⁷. A single retroviral insertion site was detected in the mMAPC and rMAPC population (Supplementary Information Table 2). Presence of the flanking region was confirmed by PCR using primers designed in the murine stem cell virus (MSCV) long terminal repeat (LTR) and in the mouse or rat genomic flanking sequence 3' of the insertion site.

We next tested the *in vitro* differentiation ability of mMAPCs and

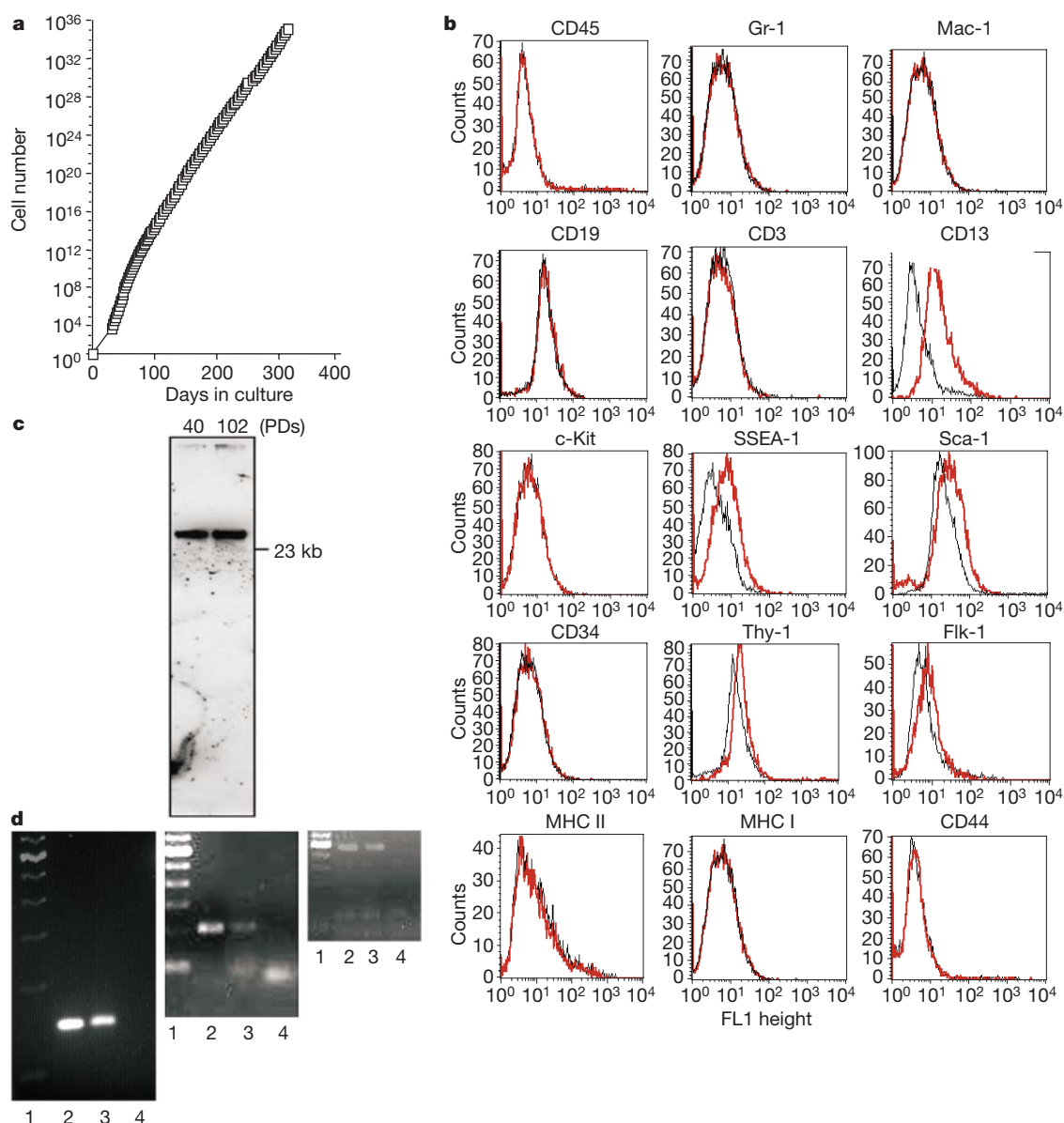


Figure 1 Characteristics of mMAPCs. Mouse BMMNCs were plated with EGF, PDGF-BB, and LIF on FN-coated plates. After 3–4 weeks, bead-selected CD45⁺/TER119⁺ cells were plated at ten cells per well in FN-coated 96-well plates in the same medium, and expanded at 0.5–1.5 × 10³ cm⁻². **a**, Cells were enumerated at each passage under a haemocytometer. **b**, mMAPCs cultured for 120 population doublings were labelled with FITC-coupled antibodies against CD45, Gr-1, Mac-1, CD19, CD3, CD13, c-Kit, SSEA-1, Sca-1, CD34, Thy-1, Flk-1, MHC class II (I-A^b), MHC class I (H-2K^b), CD44 or

immunoglobulin isotype control antibodies. Cells were analysed using a FACS-Calibur. Black line, control immunoglobulin; red line, specific antibody. **c**, Telomere length of mMAPCs cultured for 40 population doublings (PDs) (lane 1) and 102 population doublings (lane 2)²⁸. **d**, Quantitative RT-PCR for Rex-1 (left), Oct-4 (middle) and β-actin (right). Lane 1, ladder; lane 2, ES cells; lane 3, MAPCs; lane 4, no-template control (see Methods and Supplementary Information Table 1).

rMAPCs by adding cytokines chosen on the basis of what has been described for differentiation of hMAPC or ES cells to mesoderm, neuroectoderm and endoderm. Differentiation required that MAPCs be replated at $1\text{--}2 \times 10^4$ cells cm^{-2} in medium without serum, EGF, PDGF-BB and LIF, but with lineage-specific cytokines. Studies were done using two independently derived ROSA26, two C57BL/6 and one rat MAPC population expanded for 40–120 population doublings, as well as with eGFP-transduced clonal mMAPCs (repeated after 50, 80 and 120 population doublings) and clonal rMAPCs (repeated after 50 and 80 population doublings). Differentiation was similar when MAPCs cultured for 40 to more than 100 population doublings were used. No differences were seen between transduced and untransduced cells. Most data shown are therefore from the clonal eGFP⁺ mMAPCs (Fig. 3) and clonal eGFP⁺ rMAPCs (Supplementary Information Fig. 3).

As an example of mesoderm, we induced differentiation to endothelium. Undifferentiated mMAPCs or rMAPCs were negative for CD31, CD62E or von Willebrand factor (vWF) (data not shown), but expressed low levels of Flk-1 (Fig. 1b). When mMAPCs (Fig. 3) or rMAPCs (Supplementary Information Fig. 3) were cultured on fibronectin (FN) with 10 ng ml^{-1} vascular endothelial growth factor (VEGF)-B for 14 days, more than 90% of MAPCs acquired an endothelial phenotype and expressed CD31, Flk-1 and vWF. All cells staining positive for vWF also expressed eGFP, whereas a small population of eGFP⁺ ‘fibroblast-like’ cells not staining positive for vWF remained in the culture (Fig. 3a–h).

Neuroprogenitors can be expanded with PDGF-BB and induced to differentiate by removal of PDGF and addition of basic fibroblast growth factor (bFGF)³⁸. Therefore, we cultured mMAPCs (Fig. 3) and rMAPCs (Supplementary Information Fig. 3) in FN-coated

wells without PDGF-BB and EGF, but with 100 ng ml^{-1} bFGF³⁸. After 14 days, $15 \pm 4\%$ of MAPCs acquired morphologic and phenotypic characteristics of astrocytes (glial acidic fibrillary protein (GFAP)⁺), $12 \pm 3\%$ of oligodendrocytes (galactocerebroside (GalC)⁺) and $68 \pm 9\%$ of neurons (neurofilament-200 (NF-200)⁺). NF-200, GFAP or GalC were never found in the same cell. More than 90% of eGFP⁺ cells were labelled with neuroectodermal markers. Quantitative RT-PCR of mMAPCs treated with bFGF confirmed expression of neuroectodermal genes (Supplementary Information Table 3). After culture with bFGF, levels of Otx2 messenger RNA increased more than 50-fold by day 2 and became maximal by day 5. On day 4, Otx1 mRNA was upregulated 3–5-fold, and on day 5 levels of Pax2, Pax5 and nestin mRNA increased 400–800-fold over undifferentiated mMAPCs³⁹. When mMAPCs were cultured sequentially with 100 ng ml^{-1} bFGF, 10 ng ml^{-1} FGF-8 and finally 10 ng ml^{-1} brain-derived neurotrophic factor (BDNF), a more mature phenotype was seen (Fig. 3i–q). Thirty percent of cells expressed markers of dopamine-containing neurons (dopa-decarboxylase (DDC) and tyrosine hydroxylase (TH) positive), 20% of serotonin-containing (serotonin positive) neurons and 50% of γ -aminobutyric acid (GABA)-containing (GABA positive) neurons. Neuron-like cells became polarized, as Tau and MAP2 were expressed in axonal and somatodendritic compartments, respectively (Fig. 3m).

We next tested whether mMAPCs (Fig. 3) or rMAPCs (Supplementary Information Fig. 3) differentiate to endodermal cells. As described more extensively elsewhere³⁰, when replated on matrigel with 10 ng ml^{-1} FGF-4 and 20 ng ml^{-1} hepatocyte growth factor (HGF), approximately 60% of mMAPCs (Fig. 3a–h) or rMAPCs acquired epithelioid morphology and 10% of cells became binucleated. Sixty per cent of cells stained positive for albumin, cytokeratin (CK)18, and HNF-1. Figure 3a–h shows that all cells staining positive for CK18 were also eGFP⁺. We recently showed that these epithelioid cells have functional characteristics of hepatocytes, including urea and albumin production, phenobarbital-inducible p450, gluconeogenesis and low-density lipoprotein uptake³⁰.

Southern blot analyses and flanking region PCR demonstrated that differentiated eGFP⁺ mMAPCs and rMAPCs contained the identical single retroviral insert found in undifferentiated MAPCs (Fig. 2 and Supplementary Information Table 2). As 100% of differentiated cells were eGFP⁺ (Fig. 3a–h) and only a single retroviral insertion site was present, these studies show that a single MAPC differentiates into cells with morphologic, phenotypic and functional characteristics of cells representing the three germ layers.

Single MAPCs contribute to most somatic tissues

To further determine the extent of differentiation of MAPCs, we assessed their ability to contribute to various tissues by introducing MAPCs into an early blastocyst. One or 10–12 ROSA26 MAPCs obtained after 55–65 population doublings were microinjected into 3.5-day-old blastocysts of C57BL/6 mice. Blastocysts were transferred to foster mothers, and mice were allowed to develop and be born (Table 1). The number of litters born and animals per litter were in line with the birth rate seen in other studies during this period. Animals born from microinjected blastocysts were of similar size as normal animals and did not display overt abnormalities.

Chimaerism was assessed by comparing levels of Neo/ β -Gal⁴⁰ in tail clippings of 4-week-old animals with that of tissue of ROSA26 mice using Q-PCR. Chimaerism could be detected in 80% of mice derived from blastocysts in which 10–12 mMAPCs were injected and in 33% of mice derived from blastocysts microinjected with 1 mMAPC (Table 1). In both sets of animals, chimaerism ranged between 0.1% and 45%. Absence of chimaerism in some of the microinjected blastocysts may indicate that mMAPCs are not completely homogeneous. Alternatively, technical problems with injection of a single cell may be responsible for the failure of 66% of

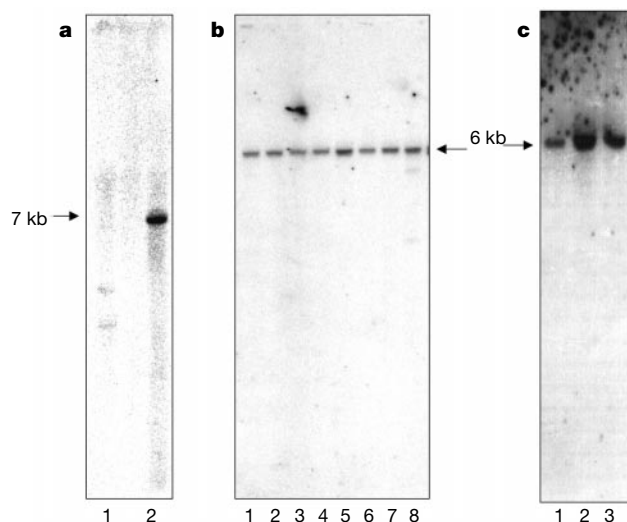


Figure 2 Single cell origin of mMAPC and rMAPC cultures initiated from ten cells per well, and their differentiated progeny (see also Supplementary Information Table 2). Mouse or rat BMMNCs, transduced with an MSCV-eGFP retrovirus⁴⁹, were depleted of CD45⁺ TER119⁺ cells, and eGFP⁺ cells were replated at ten cells per well and expanded for more than 100 population doublings. DNA from MAPCs or their differentiated progeny (Fig. 3 and Supplementary Information Fig. 3) was digested overnight with *EcoRI* or *BamHI* (cut only once in the MSCV-eGFP provirus), fragments were separated by electrophoresis, and probed with a ³²P-labelled eGFP cDNA probe. **a**, DNA extracted from pooled mMAPCs differentiated after 80 population doublings into endothelial, neuroectodermal and hepatic cells (see Fig. 3). A single retroviral insert at 7 kb was seen. **b**, Undifferentiated rMAPCs (lane 1) and seven subpopulations of rMAPCs after 75 population doublings, subcultured at 100 cells and expanded for 20 population doublings (lanes 2–8). A single retroviral insert at 6 kb was seen. **c**, rMAPCs expanded for 85 population doublings differentiated to the endothelial (lane 1), neuroectodermal (lane 2) and hepatic (lane 3) cells. A single retroviral insert at 6 kb was seen.

single mMAPCs to contribute to development of the mouse.

Animals were killed at 6–20 weeks. Some mice were frozen in liquid nitrogen, thin whole-mouse sections cut as described⁴¹, and stained with 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal). Shown in Fig. 4i is a representative animal, non-chimaeric as determined by tail clip analysis, derived from a blastocyst in which a single MAPC was injected. No X-gal staining was seen. In contrast, the animal in Fig. 4j, which was 45% chimaeric by tail clip analysis, had the contribution of a single ROSA26-derived MAPC to many somatic tissues.

We also collected multiple organs separately and determined the presence of mMAPC-derived cells by X-gal staining (Fig. 4a–h; see also Supplementary Information Fig. 4) and staining with an anti- β -gal fluorescein isothiocyanate (FITC) antibody (Fig. 5). Chimaeric animals that had Neo/ β -Gal⁺ cells, as determined by PCR in the tail clip analysis, had a contribution of the ROSA26-derived MAPC to many tissues, including brain, retina (not shown), lung, myocardium, skeletal muscle, liver, intestine, kidney, spleen, bone marrow, blood (not shown), and skin (as shown by X-gal staining, Fig. 4a–h; see also Supplementary Information Fig. 4). β -gal⁺ cells expressed markers typical for the tissue in which they had incorporated. β -gal⁺ cells in bone marrow, spleen and blood co-stained for CD45, Gr-1, Mac-1, CD19 and CD3 antigens (Fig. 5). Because of the haematopoietic chimaerism, we used triple-colour immunofluorescence to assure that β -gal⁺ cells in solid organs were not mere

haematopoietic cells. β -gal⁺ cells co-stained for pan-CK in the lung and intestine, and for CK18 in the liver (Fig. 5). We also detected CD45⁺ CK⁺ β -Gal⁺ cells in these organs. However, no cell co-stained for all three antigens (that is, β -gal, CD45 and CK). β -gal⁺ cells co-stained for dystrophin in skeletal muscle and cardiac troponin-I in the myocardium (Fig. 5). β -gal⁺ cells gave rise to neurons (Neu-N⁺) and astrocytes (GFAP⁺) throughout the entire brain, including the cortex, striatum, hippocampus, thalamus and cerebellum (Fig. 5). In the cingulate cortex, β -gal⁺ neurons and astrocytes were present, whereas in the underlying corpus callosum, β -gal⁺ astrocytes and presumed oligodendroglia were found. In the hippocampus, most of the granule cells of the dentate gyrus and pyramidal cells of the hilus were β -gal⁺, and were interspersed with β -gal⁺, MAPC-derived astrocytes.

It has been reported that after injection of ROSA26-derived neural stem cells into murine blastocysts, LacZ-expressing cells are found with varying degrees not only in the brain, but also in some mesodermal and endodermal tissues of the chimaeric mouse embryo²⁷. Our results confirm and extend these studies, as we show that single MAPCs generate balanced chimaeras, that is, contribute to most somatic cell types, and that chimaerism can be detected not only in mouse embryos, but also in mice that were 6–20 weeks old. Although some LacZ⁺ cells were seen in gonads, we have not yet performed breeding experiments to test whether MAPCs contribute to the germ line.

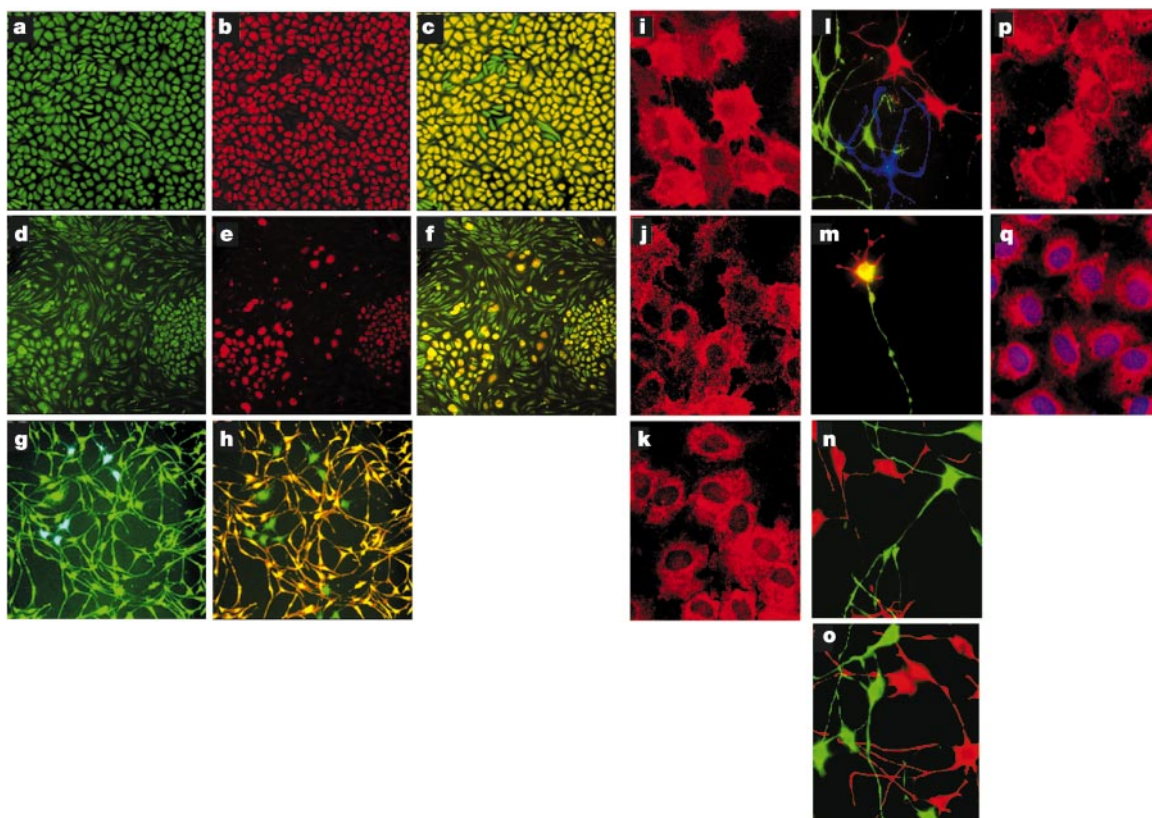


Figure 3 *In vitro* differentiation of mMAPCs to endothelium, neuroectoderm and endoderm. **a–h**, Clonal eGFP⁺ mMAPCs treated with VEGF (**a–c**), FGF-4 + HGF (**d–f**) or bFGF (**g, h**) for 14 days. Cultures were stained with anti-vWF labelled with Cy3 (**b**), anti-albumin-Cy3 (**e**), anti-GFAP-Cy5 (**g**) or anti-NF200-Cy3 (**h**). **c, f, g** and **h** show an overlay of Cy3 or Cy5 staining with eGFP. **a, c, d, f, g** and **h** show that 100% of cells were eGFP positive ($\times 10$ magnification). **i–k**, eGFP⁺ mMAPCs treated with VEGF for 14 days were stained with antibodies against CD31-Cy3 (**i**), Flk-1-Cy3 (**j**) or vWF-Cy3 (**k**) ($\times 60$ magnification). **l–o**, ROSA26 mMAPCs treated with bFGF for 14 days were stained with antibodies against GFAP-Cy3, FITC-labelled NF200 and anti-GalC-Cy5 (**l**) ($\times 20$

magnification). Alternatively, ROSA26 mMAPCs treated sequentially with bFGF, FGF-8 and BDNF were stained with antibodies against MAP2-Cy3 and FITC-labelled anti-Tau (**m**), GABA-Cy3 and FITC-labelled DDC (**n**), or TH-Cy3 and FITC-labelled serotonin (**o**) ($\times 40$ magnification). **p, q**, eGFP⁺ mMAPCs treated with FGF-4 and HGF for 14 days stained with antibodies against CK18-Cy3 (**p**), or albumin-Cy3 and HNF1-Cy5 (**q**) ($\times 60$ magnification). Colour staining: Cy3, red; FITC, green; Cy5, blue. Double positive for FITC and Cy3, yellow. In **q**, the nucleus area is positive for HNF1-Cy5 and weakly positive for Cy3, giving purple. Magnification: **a–h**, $\times 10$; **i–k**, $\times 60$; **l**, $\times 20$; **m–o**, $\times 40$; **p, q**, $\times 60$.

Table 1 Degree of chimaerism after ROSA26 MAPC injection

MAPCs per blastocyst	Litters born	No. of pups born	Neo-positive by Q-PCR (%)				
			0	1–10	10–20	20–40	>40
10–12	4 of 11	22	5 of 22 (23%)	13 of 22 (59%)	2 of 22 (9%)	1 of 22 (4.5%)	1 of 22 (4.5%)
1	3 of 5	15	8 of 15 (53%)	5 of 15 (33%)	0 of 15 (0%)	0 of 15 (0%)	2 of 15 (13%)

One or 10–12 ROSA26 MAPCs were microinjected into 116 and 38 3.5-day-old C57BL/6 blastocysts, respectively. Blastocysts were transferred to 16 foster mothers and mice were allowed to develop and be born: seven litters were born. The number of mice per litter varied between 1 and 8. After 4 weeks, chimaerism was assessed by comparing levels of Neo/ β -gal in tail clippings of 4-week-old animals with that of tissue from ROSA26 mice using Q-PCR. DNA, prepared by standard methods, underwent 40 rounds of amplification as described in Fig. 1. Primers used were Neo: 5'-TGGATTGCACGACAGTTCT-3' and 5'-TTCGCTTGGTGGTCAATG-3'. Per cent chimaerism was determined by comparing the number of Neo copies in tail samples with that in tissue from ROSA26 mice according to manufacturer's recommendations (7700 ABI PRISM Detector Software 1.6).

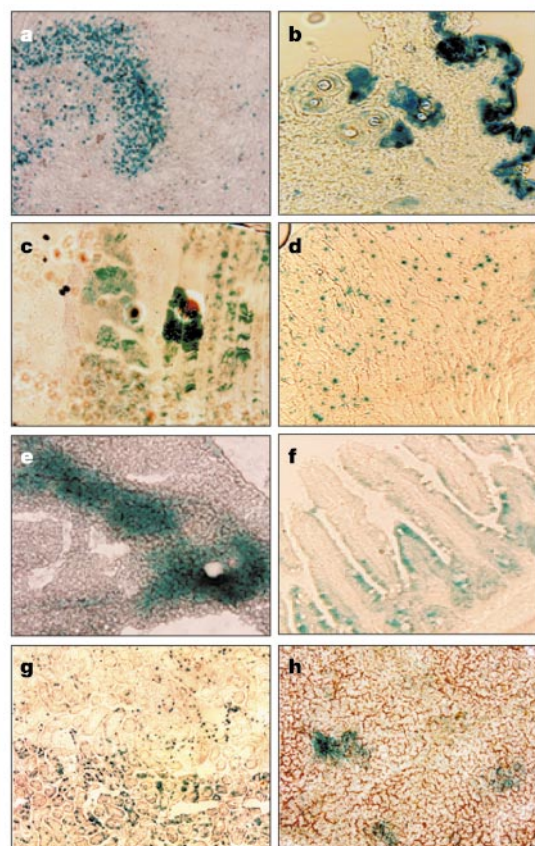


Figure 4 Chimaerism detection by X-gal staining and anti- β -gal staining in animals generated from blastocysts microinjected with a single ROSA26 MAPC (see also Table 1 and Supplementary Information Fig. 4). **a–h**, Images from X-gal-stained individual organs from a 45% chimaeric mouse, determined by Q-PCR for Neo on tail clip. Tissue sections were from: brain (**a**), skin (**b**), skeletal muscle (**c**), myocardium (**d**), liver (**e**), small intestine (**f**), kidney (**g**) and spleen (**h**). **i, j**, Images from an X-gal-stained section through a mouse that was not chimaeric (**i**) or was 45% chimaeric (**j**). Magnification, $\times 20$.

mMAPCs engraft and differentiate in tissue-specific cells

We next tested whether mMAPCs infused intravenously in post-natal animals engraft and differentiate in tissue-specific cells. To avoid rejection by recipient animals, undifferentiated ROSA26 MAPCs were injected by means of the tail vein into non-irradiated or irradiated (250 cGy) 6–8-week-old non-obese diabetic/severe combined immunodeficient (NOD/SCID) recipients. Engraftment of β -gal/Neo-containing cells was tested by immunohistochemistry (for β -gal) and Q-PCR (for Neo), 4–24 weeks after transplantation (Table 2 and Fig. 6). Engraftment, defined as more than 1% β -gal⁺ cells by immunofluorescence and/or Q-PCR, was seen in haematopoietic tissues (blood, bone marrow and spleen) and epithelium of lung, liver and intestine of all recipient animals, and was similar in animals analysed 4–24 weeks after transplantation.

β -gal⁺ cells in bone marrow and spleen co-stained for CD45 (Fig. 6). Thirty-eight to 62% of bone marrow β -gal⁺ cells co-stained for Gr-1, 9–27% for Mac-1, 18–31% for CD19, and 4–11% for TER119 (Supplementary Information Table 4). Similar results were seen for blood (not shown). No β -gal⁺ CD3⁺ T cells were seen in blood, bone marrow or spleen even though β -gal⁺ CD3⁺ T cells were present in chimaeric mice. The reason for this is unknown. Engraftment in the spleen occurred mostly as clusters of donor cells, consistent with the hypothesis that when MAPCs home to the spleen, they proliferate locally and differentiate to form a colony of donor cells, similar to colony-forming unit-spleen (CFU-S). Differentiation of mMAPCs into haematopoietic cells cannot be attributed to contamination of the mMAPCs with HSC. BMMNCs are depleted of CD45⁺ cells by column selection before MAPC cultures are initiated. MAPCs are CD3, Gr-1, Mac-1, CD19, CD34 and CD45 negative (Fig. 1), and early mesodermal or haematopoietic transcription factors, including brachyury, GATA-2 and GATA-1 (ref. 42), are not expressed in mMAPCs (complementary DNA array data; not shown). Furthermore, culture conditions for mMAPCs are not supportive for HSC and all attempts at inducing haematopoie-

Table 2 Engraftment levels in NOD/SCID mice transplanted with ROSA26 MAPCs

Animal	Time (weeks)	Radiation	Engraftment levels (%)					
			Marrow	Blood	Spleen	Liver	Lung	Intestine
1	4	No	2 (1)	2	5	7	4	2
2	5	No	3 (4)	4	5	9	5	3
3	10	No	1	3	3	6	3	2
4	16	No	4	2	3	4	3	4 (4.9)
5	24	No	3	2	3	6	4	1
6	8	Yes	8 (8)	6	4	5	2 (1.1)	7
7	8	Yes	10	8	7 (7.3)	4	6	8
8	8	Yes	5	8	3	5	5	6
9	8	Yes	7	5	5	6	4	6
10	10	Yes	5 (6)	7	9 (12.5)	5	2	8
11	11	Yes	8	8	6	5	3	10 (11.9)
12	11	Yes	6	5	4	8 (6.2)	10 (12.3)	8
SR-1	7	Yes	6	7	5	1 (1.7)	5	8
SR-2	10	Yes	5	4	8	3	4	6

Engraftment levels were determined by immunofluorescence or Q-PCR. 10^6 ROSA26 MAPCs were injected intravenously in 6–8-week-old NOD/SCID mice, maintained according to IACUC guidelines. Recipient mice were either non-irradiated or irradiated with 250 cGy (57 cGy min^{-1} by a mark 1 caesium irradiator) 16–24 before cell infusion. After 4–24 weeks, animals were killed and organs were evaluated by Q-PCR for Neo (in parenthesis) and immunohistochemistry (see Fig. 6). For SR-1 and SR-2, bone marrow from animals 9 and 10 was collected, and 1.5×10^7 cells transplanted in secondary recipients.

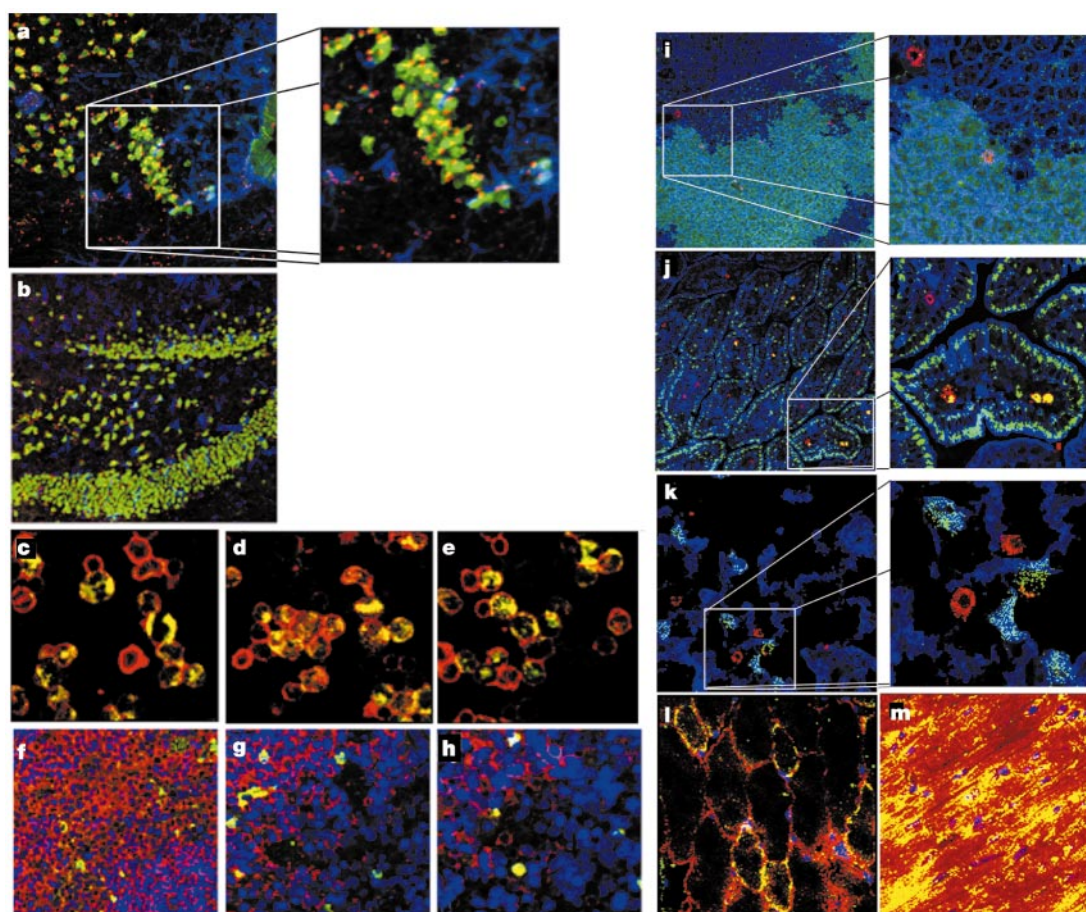


Figure 5 Immunofluorescence staining of individual organs from a 45% chimaeric mouse. **a, b**, Brain sections of cortex (**a**) and hippocampus (**b**) stained with anti- β -gal-Cy3, anti-GFAP-Cy5 and anti-NeuN-FITC. **c-e**, Bone marrow (after cytopsin centrifugation) stained with anti- β -gal-FITC and PE-coupled anti-CD45 (**c**), anti-Gr-1 (**d**) or anti-Mac-1 (**e**). **f-h**, Spleen stained with anti- β -gal-FITC and PE-coupled anti-CD45 (**f**), anti-CD3 (**g**), anti-CD19 (**h**) and TO-PRO-3. **i**, Liver stained with anti- β -gal-FITC, anti-CD45-PE and anti-CK18-Cy-5. **j**, Small intestine stained with anti- β -gal-FITC, anti-CD45-PE and

pan-CK-Cy-5. **k**, Lung stained with anti- β -gal-FITC, anti-CD45-PE and pan-CK-Cy-5. **l**, Skeletal muscle stained with anti- β -gal-FITC, anti-dystrophin-Cy3 and TO-PRO-3. **m**, Heart stained with anti- β -gal-FITC, anti-cardiac troponin-I-Cy3 and TO-PRO-3. Boxed sections are shown in greater magnification in the adjacent (right) panel. Colour staining: Cy3, red; FITC, blue; Cy5, blue; TO-PRO-3, blue. Magnification: **a**, $\times 40$; **b**, $\times 20$; **c-e**, $\times 100$; **f-h**, $\times 60$; **i-m**, $\times 20$; all insets, $\times 60$.

tic differentiation from hMAPCs *in vitro* have been unsuccessful to date²⁸.

mMAPC engraftment was also seen in liver, intestine and lung (Fig. 6). Because of the haematopoietic engraftment, we used triple-colour immunohistochemistry to discriminate between epithelial and haematopoietic cells in the same tissue sections. In the liver, β -gal⁺ CK18⁺ CD45⁻ or β -gal⁺ albumin⁺ cells formed cords of hepatocytes occupying 5–10% of a given 5- μ m section surrounding portal tracts, a pattern seen in hepatic regeneration from hepatic oval cells^{7,15}. The engraftment pattern together with the finding that only 5 of 20 sections contained donor cells, is consistent with the idea that stem cells engraft in some but not all areas of the liver, where they proliferate and differentiate into hepatocytes. Although CD45⁺ cells were identified in the same sections, no cell that stained positive for both CD45 and CK18 was seen.

In the gut, crypts contain a population of 4–5 long-lived stem cells⁵ that undergo several rounds of division in the middle and upper portions of crypts, and give rise to epithelial cells that migrate upwards, out of the crypt, onto adjacent villi. Donor-derived β -gal⁺ pan-CK⁺ CD45⁻ epithelial cells entirely covered several villi. In some villi, β -gal⁺ pan-CK⁺ CD45⁻ cells constituted only 50% of the circumference (Fig. 6r, top magnified panel), suggesting engraftment in one but not both crypts. Multiple β -gal⁺ pan-CK⁻ cells were distinctly seen in the core of intestinal villi (open arrow,

Fig. 6q). These cells co-stained for CD45 (Fig. 6r), indicating that they were donor-derived haematopoietic cells. In the lung, approximately 4% of pan-CK⁺ CD45⁻ alveolar epithelial cells were β -gal⁺. A number of recipient, pan-CK⁻ CD45⁺ β -gal⁻ haematopoietic cells can also be seen in the same section (Fig. 5t).

No contribution was seen to skeletal or cardiac muscle, tissues in which, in contrast to epithelium, little or no cell turnover is seen in the absence of tissue injury. Therefore, one may not expect significant contribution of stem cells to these tissues. Although mMAPCs differentiate into skin and tubular epithelium of the kidney when introduced in the blastocyst, we did not find engraftment in skin or kidney, in which epithelial cells undergo rapid turnover. Despite the ability of MAPCs to differentiate into neuroectoderm-like cells *ex vivo*, no significant engraftment of mMAPCs was seen in the brain, and rare donor cells found in the brain did not co-label with neuroectodermal markers. Two recent publications demonstrated that donor-derived cells with neuroectodermal characteristics were present in the brain of animals that underwent bone marrow transplantation. However, a fully ablative preparative regimen before transplantation or transplantation in newborn animals was used^{19,22}, conditions associated with breakdown of the blood–brain barrier. We infused cells in non-irradiated adult animals, or animals treated with a low dose of radiation, where the blood–brain barrier is intact or only minimally damaged. This may explain the lack of

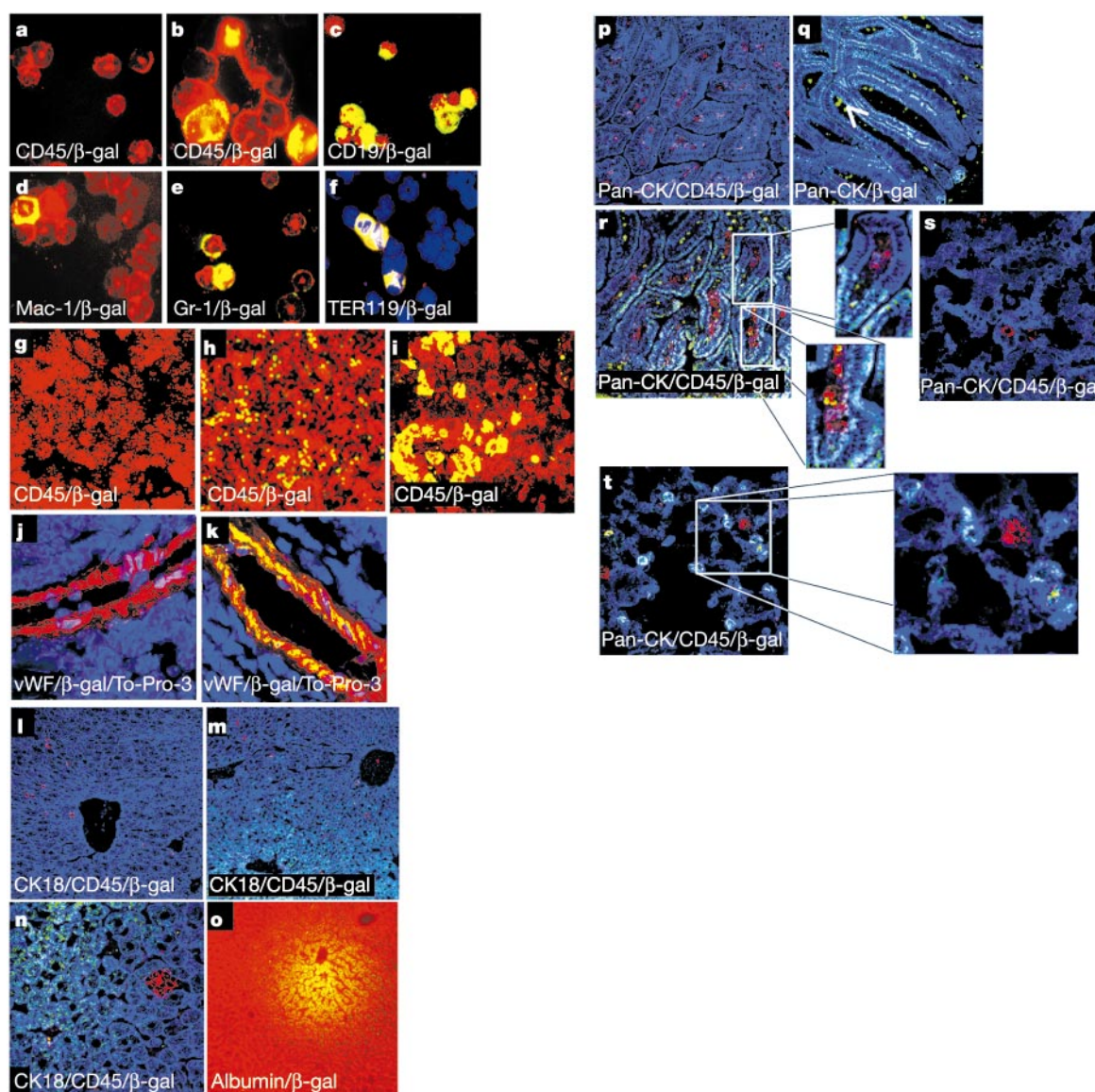


Figure 6 Engraftment and *in vivo* differentiation of mMAPCs. Tissue sections of a NOD/SCID mouse (Table 2: animal 12) 11 weeks after infusion of 10^6 ROSA26 MAPCs. **a–f**, Bone marrow (after cytospin centrifugation) of control (**a**) and study (**b–f**) animals stained with anti- β -gal-FITC antibody and PE-conjugated antibodies to CD45 (**a, b**), CD19 (**c**), Mac-1 (**d**), Gr-1 (**e**), and TER119 and DAPI (**f**). **g–i**, Spleen of control (**g**) and study animals (**h, i**) stained with anti- β -gal-FITC and anti-CD45-PE antibodies. **j, k**, Blood vessel in a control mouse (**j**) and thymic lymphoma in a study animal 16 weeks after transplantation (**k**), stained with anti- β -gal-FITC, anti-vWF-PE and TO-PRO-3. **l–o**, Liver

of control (**l**) and study animals (**m–o**) stained with anti- β -gal-FITC. **l–n** are co-stained with anti-CK18-Cy5 plus CD45-PE and **o** with anti-albumin-Cy3. **p–r**, Intestine of control mouse (**p**) and study animal (**q, r**) stained with anti- β -gal-FITC plus anti-pan-CK-Cy5. The arrow in **q** shows the most haematopoietic cell. **p** and **r** are co-stained with CD45-PE antibody. **s, t**, Lung of control (**s**) and study animal (**t**) stained with anti- β -gal-FITC plus anti-pan-CK-Cy5 plus CD45-PE antibodies. Colour staining: Cy3, red; Cy5, blue; FITC, green; TO-PRO-3, blue; DAPI, blue. Magnification: **a–f**, $\times 100$; **g, i–k, n**, $\times 60$; **l, m, p–t**, $\times 20$; **h, o**, $\times 10$; all insets, $\times 60$.

mMAPC engraftment in the central nervous system.

One animal developed a lymphoma in thymus and spleen after 16 weeks, as is commonly seen in aging NOD/SCID mice⁴³. Although the B-cell lymphoma was host-derived (CD19⁺ cells were β -gal[−]), approximately 40% of CD45[−] vWF⁺ cells in the vasculature of the tumour stained with anti- β -gal antibodies, indicating that mMAPCs can contribute functionally to neoangiogenesis *in vivo* (Fig. 6k). Higher levels of mMAPC engraftment and differentiation in radiosensitive organs—such as the haematopoietic system and intestinal epithelium (Table 2, $P < 0.001$)—after low-dose irradiation also suggests that mMAPCs may contribute functionally to host tissues. Future studies will be needed to show whether functional repopulation occurs for other tissues in the post-natal transplantation setting.

We tested whether bone marrow from primary MAPC recipients contained cells that engraft in secondary recipients. 1.5×10^7 bone marrow cells, recovered from primary recipients (Table 2: animal 11 and 12) 11 weeks after mMAPC infusion, were infused in secondary irradiated NOD/SCID recipients (Table 2, animal SR-1 and SR-2). After 7 and 10 weeks, secondary recipients were killed, and tissues were analysed for engraftment. A similar pattern of engraftment was seen in secondary recipients as in the primary recipients. Four to eight per cent of bone marrow, spleen and peripheral blood cells were β -gal⁺ CD45⁺; 6% and 8% of intestinal epithelial cells, and 4% and 5% of lung epithelial cells were β -gal⁺ pan-CK⁺ CD45[−]. However, engraftment levels in the liver of secondary recipients were lower than in the primary recipients (1% and 3% compared with 5% and 8% β -gal⁺ CK18⁺). This suggests that mMAPCs may

persist in the bone marrow of the primary recipient and differentiate into haematopoietic cells as well as epithelial cells when transferred to a second recipient.

As a control, we infused ROSA26 MAPCs grown to confluence before injection. MAPCs allowed to become confluent lose their ability to differentiate *ex vivo* in cells outside of the mesoderm, and behave like classical mesenchymal stem cells²⁸. Infusion of 10^6 confluent mMAPCs did not yield significant levels of donor cell engraftment. Although few β -gal⁺ cells were seen in bone marrow, these cells did not co-label with anti-CD45 antibodies, indicating that mesenchymal stem cells may engraft in tissues, but are no longer able to differentiate into tissue-specific cells in response to local cues.

In contrast with ES cells, we did not detect donor-derived tumours in any of the animals. Although ES cells contribute to all tissues when injected into the blastocyst, transplantation of undifferentiated ES cells leads to the development of teratomas¹, which were not seen in our model. Furthermore, intravenous infusion of ES cells does not usually lead to engraftment and tissue-specific differentiation *in vivo*, unless ES cells have been induced to commit to a lineage before transplantation.

Discussion

Three findings in our study address critically important questions in the field of stem cell plasticity⁴⁴. *In vitro* and *in vivo* conversion of bone-marrow-derived MAPCs to endothelium, ectoderm and endoderm occurs at the single cell level. Second, the blastocyst studies indicate that MAPCs contribute functionally to most somatic tissues. Third, robust, early and persistent engraftment occurred *in vivo* when MAPCs were transplanted into non-damaged recipients.

We found that MAPCs require culture conditions reminiscent of ES cells, express at least some of the genetic markers of ES cells (Oct-4, Rex-1, SSEA-1), have extensive proliferation and clonal multi-lineage differentiation potential, contribute to all organs when injected in a blastocyst, but engraft and differentiate into tissue specific cells in response to organ-specific cues. Cell fusion has recently been suggested as an explanation for stem cell plasticity. In two studies somatic cells could be induced to fuse with ES cells *in vitro*, generating tetraploid cells with ES-like characteristics^{45,46}. Our *in vitro* studies demonstrating that single euploid MAPCs—never co-cultured with tissue-specific cells or ES cells—differentiate into cells of the three germ layers, show that the *in vitro* behaviour of MAPCs cannot be attributed to stem cell fusion. Although no definitive studies were done *in vivo* to exclude the possibility that cell fusion is responsible for the differentiation in multiple tissues, the high frequency with which chimaerism was observed and the balanced chimaerism differs from what was shown by ref. 46. Finally, the speed and robustness with which engraftment and tissue-specific differentiation is seen in animals without need for selectable pressure, also argues against the idea that MAPC engraftment and differentiation in post-natal animals is caused by fusion.

A second possibility is that pluripotent stem cells persist even after birth in multiple organs, and that when stimulated, they proliferate and differentiate in response to local cues provided by the organ they are recruited to. A third possibility is that a tissue-specific stem cell may undergo genetic re-programming in culture similar to that which occurs in the 'cloning process'^{47,48}. Monthly cytogenetics²⁸ of mMAPCs and rMAPCs did not reveal abnormalities, except in one mMAPC population that became hyperdiploid at 45 population doublings, and was no longer used for studies. When mMAPCs or rMAPCs were grown to confluency, they stopped proliferating, and when cultured in serum-free medium and differentiation-inducing cytokines after 40 or more than 120 population doublings, growth arrest and terminal differentiation was seen. Furthermore, no tumour formation was seen in immunodeficient mice that received mMAPCs intravenously. Thus, even

though re-programming may have occurred *in vitro* under the MAPC culture conditions, we have no evidence that transformation occurred.

Irrespective of their origin, MAPCs hold great promise for the treatment of degenerative or inherited diseases. Similar to ES cells, allogeneic MAPCs may be used to correct degenerative or congenital diseases. MAPCs differentiate into haematopoietic cells *in vivo* and can thus be used to establish haematopoietic chimaerism, which should make such an allogeneic cell therapy approach feasible. In contrast to ES cells, MAPCs can be selected from autologous bone marrow and used undifferentiated or after genetic manipulation in local or systemic therapies. Furthermore, absence of teratoma formation when undifferentiated MAPCs are infused should allow the use of undifferentiated MAPCs to treat systemic diseases, such as inherited enzyme deficiencies or muscular dystrophy. □

Methods

Antibodies used

Antibodies against NF-200 (clone N52, 1:400), GalC (G-9152; 1:100), CK18 (C-8541; 1:300), pan-CK (C-2562; 1:100), albumin (A-6684; 1:100), GABA (A-2052, 1:500), MAP2 (AP20, 1:400), dopa-decarboxylase (DDC-109, 1:100), tyrosine hydroxylase (TH-16, 1:1,000), serotonin (S-5545, 1:1,000), cardiac troponin-I (sc-8118, 1:100), and dystrophin (D-8168; 1:100) were from Sigma. Polyclonal antibodies against Tau, vWF, Flk-1 and hepatocyte nuclear factor (HNF)-1 were from Santa Cruz Biotechnology Inc. Antibody against GFAP was from Santa Cruz Biotechnology or DAKO Corporation. Antibody against CD31 was from BD PharMingen. Control mouse, rabbit or goat immunoglobulin- γ and FITC, Cy3-labelled secondary antibodies were from Sigma; Cy5-labelled antibodies were from Chemicon International. Donkey anti-NeuN (1:100), anti-GFAP (1:500), and anti- β -gal (1:2,000) were from Chemicon, Sigma and Cortex Biochem, respectively. Secondary anti-donkey antibodies (FITC, 1:200; Cy-3, 1:400; Cy-5, 1:200) were from Jackson Immunoresearch. Anti- β -gal-FITC antibody was from Rockland Immunochemicals. FITC- or phycoerythrin (PE)-coupled antibodies against CD45, Gr-1, Mac-1, CD19, CD3, CD13, c-Kit, Sca-1, CD34, Thy-1, Flk-1, MHC class I (H-2K^b), MHC class II (I-A^b), and CD44 were from BD PharMingen, and anti-SSEA-1 antibody was from the Developmental Studies Hybridoma Bank.

Differentiation culture and analysis

Clonal eGFP⁺ or ROSA26 mMAPCs were replated in 60% DMEM-LG, 40% MCDB-201, ITS, LA-BSA, 10^{-9} M dexamethasone, 10^{-4} M ascorbic acid 2-phosphate, 100 U penicillin and 1,000 U streptomycin^{28,29}. Endothelial differentiation was induced with VEGF as described^{28,29}. For neuroectodermal differentiation, 1×10^4 mMAPCs per cm² were plated on FN with 100 ng ml⁻¹ bFGF (R&D Systems). Alternatively, cells were treated sequentially with 100 ng ml⁻¹ bFGF for 7 days, 10 ng ml⁻¹ FGF-8 for 7 days and 10 ng ml⁻¹ BDNF for 7 days (R&D Systems). Hepatocyte differentiation was induced as described³⁰. Cells were fixed with 4% paraformaldehyde and methanol at room temperature, and incubated sequentially for 30 min each with primary and secondary antibodies. Between steps, slides were washed with PBS/BSA. Cells were examined by confocal fluorescence microscopy (Confocal 1024 microscope; Olympus AX70, Olympus Optical Co. Ltd). To assess the frequency of different cell types in a given culture, we counted the number of cells staining positive with a given antibody in four low-power visual fields (200–500 cells per field).

Tissue collection and analysis

For the whole-mouse mount, 10- μ m whole-body sections near the midline were prepared as described⁴¹. Tissue sections were stained for β -galactosidase enzyme activity with the β -gal staining kit from Invitrogen at pH 7.4. Manufacturer's instructions were followed except for fixation, for which the tissue sections were incubated for 5 min instead of 10 min.

A total of 0.5–1 ml of blood was obtained when animals were killed, and bone marrow was collected by flushing femurs and tibias. For phenotyping, red blood cells and bone marrow were depleted using ice-cold ammonium chloride (Stem Cell Technologies Inc.) and 10^5 cells were used for cytospin centrifugation. For serial transplantation, 1.5×10^7 cells from two femurs and two tibias were transplanted into individual secondary recipients by means of tail vein injection. Cytospin specimens of blood and bone marrow were fixed with acetone for 10 min at room temperature.

Lungs were inflated with 1 ml 1:4 dilution of optimum cutting temperature (OCT) compound (Sakura-Finetek Inc) in PBS. Specimens of spleen, liver, lung, intestine, skeletal muscle, myocardium, kidney and brain of the recipient animals were collected and cryopreserved in OCT at -80°C and in RNA Later (Ambion Inc.) at -20°C for Q-PCR. Five-micrometre-thick fresh frozen sections were mounted on glass slides and fixed in acetone for 10 min at room temperature. After incubation with isotype sera for 20 min, slides were stained sequentially with antibodies against cell-type antigens, anti- β -gal antibody, in some instances antibodies against CD45, or a nuclear counter stain (4,6-diamidino-2-phenylindole (DAPI) or TO-PRO-3).

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1. Thomson, J. A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147 (1998).
2. Frankel, M. S. In search of stem cell policy. *Science* **298**, 1397 (2000).
3. Weissman, I. L. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *Science* **287**, 1442–1446 (2000).
4. Gage, F. H. Mammalian neural stem cells. *Science* **287**, 1433–1438 (2000).
5. Potten, C. Stem cells in gastrointestinal epithelium: numbers, characteristics and death. *Phil. Trans. R. Soc. Lond. B* **353**, 821–830 (1998).
6. Watt, F. Epidermal stem cells: markers patterning and the control of stem cell fate. *Phil. Trans. R. Soc. Lond. B* **353**, 831 (1997).
7. Alison, M. & Sarraf, C. Hepatic stem cells. *J. Hepatol.* **29**, 678–683 (1998).
8. Pittenger, M. F. *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143–147 (1999).
9. Ferrari, G. *et al.* Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* **279**, 528–530 (1998).
10. Gussoni, E. *et al.* Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* **401**, 390–394 (1999).
11. Orlic, D. *et al.* Bone marrow cells regenerate infarcted myocardium. *Nature* **401**, 701–705 (2001).
12. Jackson, K. *et al.* Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J. Clin. Invest.* **107**, 1395–1402 (2001).
13. Lin, Y., Weisdorf, D. J., Solovey, A. & Hebbel, R. P. Origins of circulating endothelial cells and endothelial outgrowth from blood. *J. Clin. Invest.* **105**, 71–77 (2000).
14. Asahara, T. *et al.* Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ. Res.* **85**, 221–228 (1999).
15. Petersen, B. E. *et al.* Bone marrow as a potential source of hepatic oval cells. *Science* **284**, 1168–1170 (1999).
16. Theise, N. D. *et al.* Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. *Hepatology* **31**, 235–240 (2000).
17. Lagasse, E. *et al.* Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. *Nature Med.* **6**, 1229–1234 (2000).
18. Krause, D. S. *et al.* Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* **105**, 369–377 (2001).
19. Brazelton, T. R., Rossi, F. M. V., Keshet, G. I. & Blau, H. E. From marrow to brain: expression of neuronal phenotypes in adult mice. *Science* **290**, 1775–1779 (2000).
20. Kopen, G., Prockop, D. & Phinney, D. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc. Natl Acad. Sci. USA* **96**, 10711–10716 (1999).
21. Mezey, E., Chandross, K. J., Harta, G., Maki, R. A. & Mckercher, S. R. Turning blood into brain: cells bearing neuronal antigens generated *in vivo* from bone marrow. *Science* **290**, 1779–1782 (2000).
22. Sanchez-Ramos, J. *et al.* Adult bone marrow stromal cells differentiate into neural cells in vitro. *Exp. Neurol.* **164**, 247–256 (2000).
23. Bjornson, C., Rietze, R., Reynolds, B., Magli, M. & Vescovi, A. Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells *in vivo*. *Science* **283**, 354–357 (1999).
24. Morshead, C. M., Benveniste, P., Iscoe, N. N. & van der Kooy, D. Hematopoietic competence is a rare property of neural stem cells that may depend on genetic and epigenetic alterations. *Nature Med.* **8**, 268–273 (2002).
25. Jackson, K., Mi, T. & Goodell, M. A. Hematopoietic potential of stem cells isolated from murine skeletal muscle. *Proc. Natl Acad. Sci. USA* **96**, 14482–14486 (1999).
26. Kawada, H. & Ogawa, M. Bone marrow origin of hematopoietic progenitors and stem cells in murine muscle. *Blood* **98**, 2008–2013 (2001).
27. Clarke, D. L. *et al.* Generalized potential of adult neural stem cells. *Science* **288**, 1660–1663 (2000).
28. Reyes, M. *et al.* Purification and *ex vivo* expansion of postnatal human marrow mesodermal progenitor cells. *Blood* **98**, 2615–2625 (2001).
29. Reyes, M. *et al.* Origin of endothelial progenitors in human post-natal bone marrow. *J. Clin. Invest.* **109**, 337–346 (2002).
30. Schwartz, R. E. *et al.* Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. *J. Clin. Invest.* **109**, 1291–1302 (2002).
31. Odorico, J. S., Kaufman, D. S. & Thomson, J. A. Multilineage differentiation from human embryonic stem cell lines. *Stem Cells* **19**, 193–204 (2001).
32. Williams, R. L. *et al.* Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature* **336**, 684–687 (1988).
33. Scholer, H. R., Hatzopoulos, A. K., Balling, R., Suzuki, N. & Gruss, P. A family of octamer-specific proteins present during mouse embryogenesis: evidence for germline-specific expression of an Oct factor. *EMBO J.* **8**, 2543–2550 (1989).
34. Ben-Shushan, E., Thompson, J. R., Gudas, L. J. & Bergman, Y. Rex-1, a gene encoding a transcription factor expressed in the early embryo, is regulated via Oct-3/4 and Oct-6 binding to an octamer site and a novel protein, Rox-1, binding to an adjacent site. *Mol. Cell Biol.* **18**, 1866–1878 (1998).
35. Jordan, C., McKearn, J. & Lemischka, I. Cellular and developmental properties of fetal hematopoietic stem cells. *Cell* **61**, 953–963 (1990).
36. Nolte, J., Dao, M., Wells, S., Smogorzewska, E. & Kohn, D. Transduction of pluripotent human hematopoietic stem cells demonstrated by clonal analysis after engraftment in immune-deficient mice. *Proc. Natl Acad. Sci. USA* **93**, 2414–2419 (1996).
37. Lenvik, T., Lund, T. C. & Verfaillie, C. M. Blockerette-ligated capture T7 amplified RT-PCR, a new method for determining flanking sequences. *Mol. Therapy* (in the press).
38. Palmer, T. D., Markakis, E. A., Willhoite, A. R., Safar, F. & Gage, F. H. Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *J. Neurosci.* **19**, 8487–8497 (1999).
39. Lee, S. H., Lumelsky, N., Studer, L., Auerbach, J. M. & McKay, R. D. Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nature Biotechnol.* **18**, 675–679 (2000).
40. Zambrowicz, B. P. *et al.* Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc. Natl Acad. Sci. USA* **94**, 3789–3794 (1997).
41. Reinhardt, R. L., Khoruts, A., Merica, R., Zell, T. & Jenkins, M. K. Visualizing the generation of memory CD4 T cells in the whole body. *Nature* **401**, 101–105 (2001).
42. Weiss, M. J. & Orkin, S. H. GATA transcription factors: key regulators of hematopoiesis. *Exp. Hematol.* **23**, 99–107 (1995).
43. Prochazka, M., Gaskins, H. R., Shultz, L. D. & Leiter, E. H. The nonobese diabetic scid mouse: model for spontaneous thymomagenesis associated with immunodeficiency. *Proc. Natl Acad. Sci. USA* **89**, 3290–3294 (1992).
44. Anderson, D. J., Gage, F. H. & Weissman, I. L. Can stem cells cross lineage boundaries? *Nature Med.* **7**, 393–395 (2001).
45. Terada, N. *et al.* Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature* **416**, 542–545 (2002); advance online publication, 13 March 2002 (doi:10.1038/nature00730).
46. Ying, Q. Y., Nichols, J., Evans, E. P. & Smith, A. G. Changing potency by spontaneous fusion. *Nature* **416**, 545–548 (2002); advance online publication, 13 March 2002 (doi:10.1038/nature729).
47. Rideout, W. M. *et al.* Generation of mice from wild-type and targeted ES cells by nuclear cloning. *Nature Genet.* **24**, 109–110 (2000).
48. Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J. & Campbell, K. H. Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**, 810–813 (1997).
49. Pear, W. S. *et al.* Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. *Blood* **92**, 3780–3792 (1998).

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Competing interests statement

The authors declare competing financial interests: details accompany the paper on *Nature's* website (<http://www.nature.com/nature>).

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Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease

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Parkinson's disease is a widespread condition caused by the loss of midbrain neurons that synthesize the neurotransmitter dopamine. Cells derived from the fetal midbrain can modify the course of the disease, but they are an inadequate source of dopamine-synthesizing neurons because their ability to generate these neurons is unstable. In contrast, embryonic stem (ES) cells proliferate extensively and can generate dopamine neurons. If ES cells are to become the basis for cell therapies, we must develop methods of enriching for the cell of interest and demonstrate that these cells show functions that will assist in treating the disease. Here we show that a highly enriched population of midbrain neural stem cells can be derived from mouse ES cells. The dopamine neurons generated by these stem cells show electrophysiological and behavioural properties expected of neurons from the midbrain. Our results encourage the use of ES cells in cell-replacement therapy for Parkinson's disease.

Fetal midbrain precursors can proliferate and differentiate into dopamine-synthesizing neurons *in vitro*, and transplantation of these cells leads to recovery in a rat model of Parkinson's disease^{1,2}. However, these precursor cells, which are derived from either rodent or human midbrain, generate dopamine neurons for only short periods in culture. ES cells can proliferate extensively in an undifferentiated state and may provide an unlimited source of many cell types. A related benefit of using ES cells is their accessibility for genetic engineering, which will permit the isolation and functional analysis of specific cell types. The isolation of human ES cells and the related embryonic germ cells has stimulated interest in their potential clinical value^{3,4}. Although the use of ES cells in cell therapy is widely discussed, there are few cases showing that ES cell technology can be successfully applied to animal models of disease⁵⁻⁷. We have defined signals that improve the efficiency of dopamine-neuronal differentiation from ES cells, but this approach may still provide too few neurons for widespread use⁸. The purpose of our study here was to develop a method of further increasing the efficiency of midbrain-specific generation of dopamine neurons from ES cells, and to demonstrate that these cells can functionally integrate into host tissue as well as lead to recovery in a rodent model of Parkinson's disease.

Generation of midbrain CNS precursors

Previously, we developed a five-stage method that leads to the efficient differentiation of ES cells into neurons^{8,9}. Nuclear receptor related-1 (Nurr1) is a transcription factor that has a role in the differentiation of midbrain precursors into dopamine neurons¹⁰⁻¹³. We used a cytomegalovirus plasmid (pCMV) driving expression of a rat Nurr1 complementary DNA modified to express an antigenic site derived from the haemagglutinin protein (HA) to establish stable Nurr1 ES cell lines. Nurr1 ES cells were processed through the five-stage differentiation method⁸. Immunoblotting was used in stages 4 and 5 using anti-Nurr1 or anti-HA antibodies. The anti-HA

antibody shows that the introduced gene is expressed at high levels at stage 4 but at much lower levels at stage 5. In contrast, the endogenous Nurr1 gene was expressed at low levels in stage 4 cells but at higher levels after differentiation at stage 5 (Fig. 1a). These cells differentiated appropriately to cells positive for tyrosine hydroxylase (TH) (Fig. 1a and b). The subcellular localization of Nurr1 was monitored by immunocytochemistry using anti-HA antibody. In undifferentiated ES cells, Nurr1 was located in a restricted site in the nucleus (Fig. 1c). To assess whether Nurr1 induces TH expression in the appropriate neural cell types, we used double labelling for TH and specific molecular markers for neurons and glia. All TH⁺ cells were double labelled with the neuronal marker Tuj1 but not with the markers for glial lineages (data not shown), indicating that the expression of the Nurr1 gene induced TH expression specifically in neurons. We found no evidence of elevated rates of cell death in Nurr1-expressing ES or neural stem cells (data not shown), ruling out that expression of transfected Nurr1 alters the distribution of cell fates by causing the death of specific cell types. Also, Nurr1 expression alone could not switch the regional identity of central nervous system (CNS) precursors, as evidenced by the lack of Engrailed 1 (En-1) expression in Nurr1-transfected cortical CNS stem cells positive for Bfl (a forebrain-specific marker) (data not shown). These results suggest that the TH⁺ neurons derived from Nurr1 ES cells are generated from precursor cells that are readily responsive to the actions of the Nurr1 protein.

Modification of the above procedure can differentiate ES cells into insulin-secreting structures similar to pancreatic islets¹⁴. This directly raises the important problem of enriching for specific cell types when using ES cells as a starting point. Cells at early stages of vertebrate development are thought to adopt mesodermal and ectodermal differentiation paths as alternate fates. Leukaemia inhibitory factor (LIF) inhibits the formation of cardiac mesoderm¹⁵ and may promote neuronal differentiation at early stages of CNS development¹⁶. The pancreatic and duodenal homeobox-1 (Pdx-1) gene is an early regulator of the differentiation of cells in the endocrine pancreas¹⁷. LIF treatment of stage 2 cell aggregates strongly reduces the expression of Pdx-1 and promotes the differentiation of neurons.

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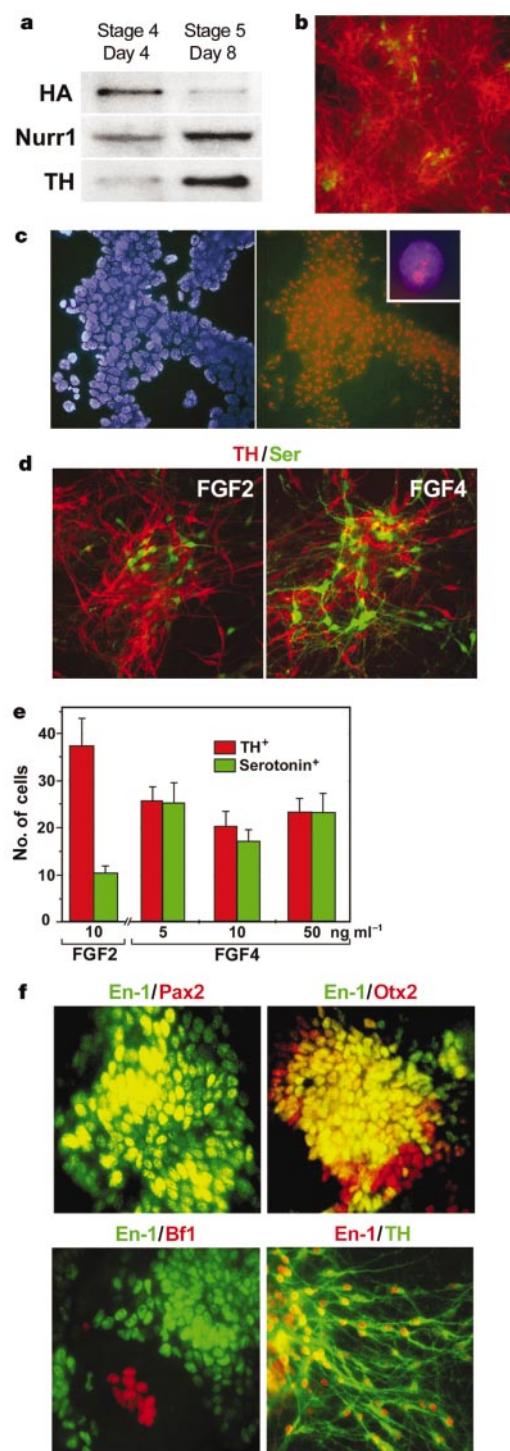


Figure 1 Properties of neural precursors derived from Nurr1 ES cells. **a**, Immunoblot analysis for transfected Nurr1 (HA), total Nurr1 and TH at the end of stage 4 and at day 8 of stage 5. **b**, Differentiation of Nurr1-transfected ES cells into TH⁺ (red) and serotonin⁺ (green) neurons at day 10 of stage 5. The proportion of TH⁺ cells was greater than that in wild-type ES cells. **c**, Nurr1 overexpression was evaluated using anti-HA immunostaining (red) at stage 1. DAPI (4,6-diamidino-2-phenylindole), blue; TH, green. Inset, co-localization of Nurr1 with DAPI at higher magnification. **d**, Stage 4 cells were cultured in the presence of Shh (500 ng ml⁻¹) and FGF8 (100 ng ml⁻¹) with 20 ng ml⁻¹ of FGF2 or FGF4 (5, 10 and 50 ng ml⁻¹) and immunostained with TH and serotonin at day 10 of stage 5. **e**, The yield of TH⁺ and serotonin⁺ neurons/ low power field (SEM from 40 different fields of view). **f**, Nurr1-expressing stage 4 cells show markers of midbrain precursors. En-1⁺ immunoreactivity co-localizes with Pax2 and Otx2. In contrast, few Bf1⁺ cells were positive for En-1. After differentiation at stage 5, most TH⁺ neurons expressed the midbrain-specific marker En-1.

In the developing CNS, midbrain dopamine-producing and hindbrain serotonin-producing neurons are generated on either side of the isthmus organizer, a signalling centre known to influence neuronal differentiation^{18,19}. Both of these neuronal types are dependent on fibroblast growth factor 8 (FGF8) and sonic hedgehog (Shh) synthesized by the isthmus organizer^{18,19}. In addition, precursors for serotonin-positive neurons in the ventral hindbrain are sensitive to FGF4 (ref. 20). The number of dopamine neurons generated from ES cells can be significantly increased when treating cells with FGF8 and Shh⁸. Therefore, we assessed the ability of FGF4 to generate serotonin neurons from ES cells by substituting FGF2 with FGF4 during stage 4. Stage 4 cells treated with FGF4 for 2 days and exposed to Shh and FGF8 for a further 4 days were analysed by immunocytochemistry after differentiation in stage 5 (Fig. 1d). FGF4 promoted a 2.5-fold increase in serotonin neurons and reduced the population of TH⁺ cells (Fig. 1e). This result indicates that, as with endogenous mid- and hindbrain CNS precursors, the cell population at stage 4 is responsive to developmentally appropriate signals generated by the isthmus organizer.

To further define the cells present at stage 4, we evaluated the expression of transcription factors that characterize precursors in different regions of the CNS. Previous studies have shown that Otx2 expression occurs in the fore- and midbrain with a posterior limit at the isthmus organizer^{21,22}. Pax2 is expressed in the mid- and hindbrain before becoming restricted to hindbrain cells^{21,22}. At

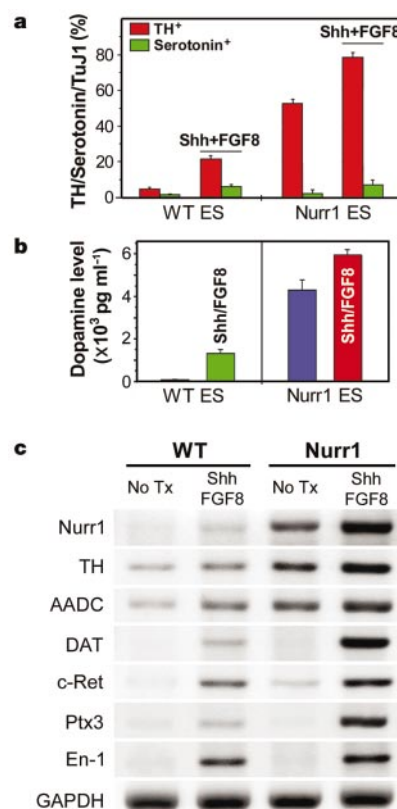


Figure 2 Characterization of TH⁺ neurons derived from Nurr1-transfected ES cells. **a**, Wild-type (WT) and Nurr1 ES cells were induced to differentiate by the five-stage protocol described in the text. The generation of TH⁺ and serotonin⁺ neurons was measured at day 10 of stage 5. The generation of TH⁺ neurons is enhanced in wild-type and Nurr1 ES cells by treatment with Shh and FGF8 in stage 4. **b**, HPLC quantification of dopamine release by stage 5 ES cells after depolarization in HBSS (56 mM KCl) for 15 min. Data are shown as mean $\pm$ s.e.m. **c**, Expression analysis of genes involved in midbrain neuron development and function by RT-PCR in stage 5. Note that midbrain-specific genes *Nurr1*, *Ptx3*, *En-1* and the dopamine transporter (*DAT*) are expressed at low levels in the absence of Nurr1 overexpression and Shh/FGF8 treatment at stage 4.

stage 4, many cells positive for En-1 co-express Pax2 (65.5%) and Otx2 (80%) (Fig. 1f). Less than 1% of the En-1⁺ cells label with Bfl (ref. 23 and Fig. 1f). No cells expressing motor neuron transcription factors HB9 or Isl1 were seen at either stage 4 or 5 (data not shown). Also, similar to En-1 expression patterns seen in postmitotic differentiated dopamine neurons^{10,24}, nearly all ES-derived TH⁺ neurons expressed En-1 in their nucleus (Fig. 1f). These results strongly support the view that midbrain precursors and differentiated neurons can be efficiently generated from ES cells.

Functional characterization of dopamine neurons

In ES cells, Nurr1 overexpression alone promoted an increase in the proportion of TH⁺ neurons from 5 to 50% (Fig. 2a). When Shh and FGF8 were present at stage 4, the proportion of TH⁺ neurons generated from Nurr1 ES cells could be increased to 78% (mean $\pm$ s.e.m., 78.2 ± 3.2 ; Fig. 2a). In Nurr1 ES cells, serotonin-positive neurons were increased 2.3-fold by the treatment of Shh and FGF8, but this value is not significantly different from that in wild-type (WT) ES cells, indicating that the differentiation of serotonin neurons was not affected by Nurr1 (Fig. 2a). This is consistent

with *in vivo* data showing that deletion of Nurr1 has no effect on the serotonin neurons¹³ and emphasizes that Nurr1 must act cooperatively with other signals specifically found in midbrain cells.

The capacity of the TH⁺ neurons to synthesize and release dopamine was assessed by reverse-phase high-performance liquid chromatography (HPLC). The dopamine released by depolarization was markedly elevated in the cultures of stage 5 Nurr1 ES cells compared with WT ES cells (Fig. 2b). The presence of noradrenaline and adrenaline was not observed in these cultures using an assay with a detection limit of 70 pg ml⁻¹ and 120 pg ml⁻¹ for noradrenaline and adrenaline, respectively.

To further characterize the dopamine-producing TH⁺ neurons, semi-quantitative polymerase chain reaction with reverse transcription (RT-PCR) was used to evaluate expression of specific genes involved in dopamine neuron development and function. Nurr1, TH and aromatic L-amino acid decarboxylase (AADC) were all upregulated in differentiated Nurr1 ES cells compared with WT ES cells (Fig. 2c). Dopamine β -hydroxylase (DBH) and the transcription factor Phox2a, selective markers for adrenaline-mediated neurons, were not detected (data not shown). Treatment with Shh and FGF8 increased the expression of dopamine transporter (DAT),

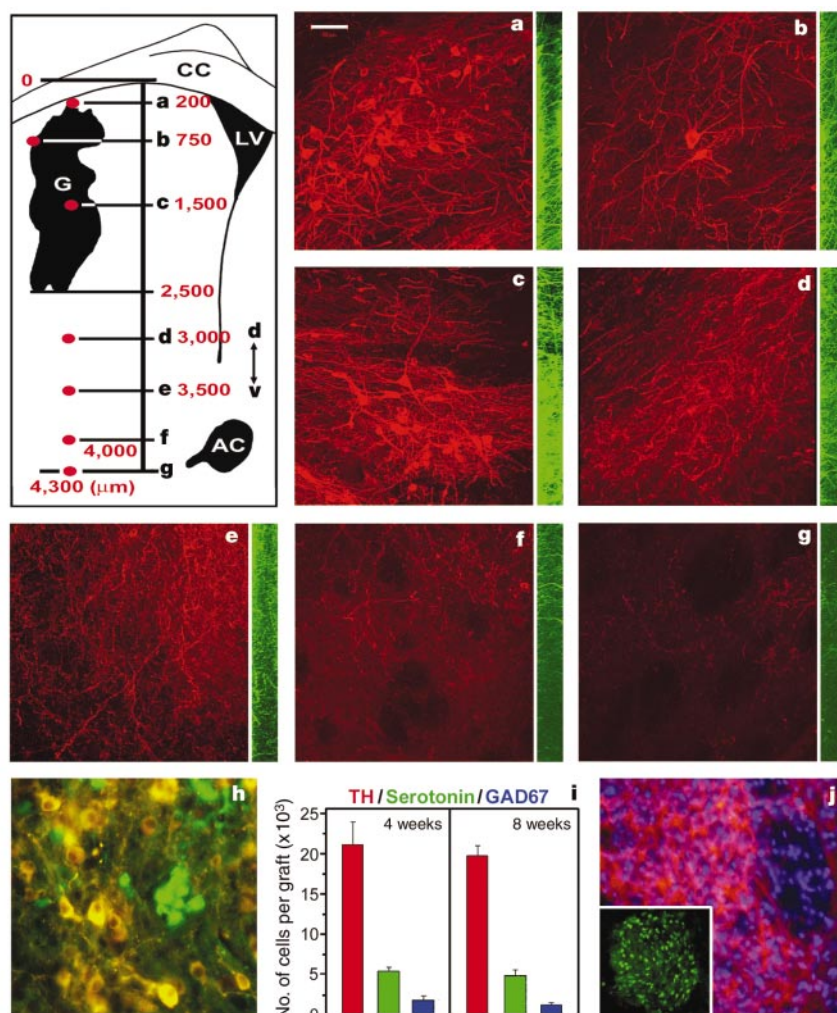


Figure 3 Nurr1 ES cells integrate into the striatum of hemiparkinsonian rats. The diagram shows a drawing of a single section through a graft (G) in the striatum (LV, lateral ventricle; AC, anterior commissure). Single confocal images after immunohistochemistry for TH (red) are shown (a–g) from regions marked by red dots in the diagram. The distribution of cells and processes through the thickness of the section (35 μ m) is shown by the z-series displayed in green on the right. Note the many TH⁺ processes that extend away from the

graft into the parenchyma of the host striatum (d–f). Scale bar, 50 μ m.

h, Immunoreactivity for the mouse-specific antigen M2 (green) and for TH (red, or yellow where it overlaps green) in the dorsal striatum of grafted animals. **i**, Survival of TH⁺, serotonin⁺ and GAD67⁺ cells in grafts at 4 and 8 weeks after transplantation. **j**, Lack of immunostaining for Ki-67 (green) in a graft (M2, red). Inset shows Ki-67 staining of a rat brain section containing glioma cells as a positive control.

Table 1 Electrophysiological characterization of TH⁺ neurons

	TH ⁺ [A]	TH ⁺ (graft) [B]	TH ⁺ (host) [C]
Resting membrane potential (mV)*	-64 ± 3.5 (15) ^C	-67 ± 2.9 (21)	-77 ± 1.6 (19) ^A
Rectifier ratio (early/late)*	0.81 ± 0.05 (15) ^{B,C}	0.97 ± 0.02 (20) ^A	0.99 ± 0.03 (17) ^A
Action potential duration (ms)*	2.23 ± 0.17 (15) ^C	1.47 ± 0.24 (20)	1.1 ± 0.21 (17) ^A
Action potential amplitude (mV)	60 ± 2.1 (15)	57 ± 3.2 (20)	62 ± 2.2 (17)
Current–frequency relation (Hz pA ⁻¹)*	0.18 ± 0.11 (13) ^{B,C}	0.98 ± 0.14 (20) ^A	1.3 ± 0.21 (15) ^A
Maximum firing frequency (Hz)*	47 ± 8.4 (11) ^{B,C}	69 ± 6.5 (20) ^A	81 ± 6.7 (15) ^A

Characteristics of TH⁺ versus TH⁺ neurons *in vivo*. There are several properties of TH⁺ neurons, characteristic of dopamine neurons, that are significantly different from those of TH⁺ neurons. These differences are phenotype dependent, regardless of the origin of the cell—that is, host derived rather than ES cell derived. Data are presented as mean ± s.e.m. The number in parentheses is the number of cells for each value. Letters in brackets represent the different groups.

**P* ≤ 0.025 by analysis of variance. A superscript letter denotes that the value is significantly different from the indicated group, using Tukey's post-hoc analysis.

which in vertebrates is exclusively found in dopamine neurons (Fig. 2c). The homeobox gene *Ptx3*, uniquely found in midbrain dopamine neurons²⁵, was highly expressed in cultures of Nurr1 ES cells in the presence of Shh and FGF8 (Fig. 2c). A high level of *En-1* messenger RNA was also found in Shh/FGF8-treated cells, suggesting that exposure of precursor cells to specific factors regulates gene expression at later stages of neuronal differentiation. These results are strong evidence that TH⁺ cells generated from Nurr1 ES cells express many molecular, morphological and functional features expected of midbrain dopamine neurons.

We next explored the ability of differentiated WT and Nurr1-transfected ES cells to survive, integrate, and function in host animals. In rodents, administration of 6-hydroxy dopamine (6-OH-DA) in the striatum kills dopamine neurons, providing a useful model of Parkinson's disease. Animals lesioned with 6-OH-DA either received a sham operation or a graft of 5 × 10⁵ Nurr1 ES cells (day 3 of stage 5). The sham-grafted animals showed no TH⁺

elements in the ipsilateral substantia nigra or the striatum. Animals grafted with Nurr1 ES cells were used for morphological analysis by TH immunostaining. All grafts were easily detected in the dorsal striatum by staining for the mouse-specific surface antigen M2 and for TH. TH⁺ cells in the grafts show complex morphologies (Fig. 3a–c) and are positive for the calcium-binding protein calbindin (relative molecular mass 28,000), which is normally expressed in a subset of midbrain dopamine neurons that project to the striatum²⁶ (data not shown). In serial sections, the grafts were found to extend across 2.5 mm of the host striatum. TH⁺ cell bodies were restricted to the region of the graft (Fig. 3a–c) but TH⁺ processes were found in the parenchyma of the host striatum up to 2 mm from the graft (Fig. 3d–g). Many of the M2⁺ grafted cells also expressed TH (Fig. 3h). To characterize the phenotype of grafted cells, we measured the number of neurons positive for TH, serotonin and glutamate decarboxylase (GAD67) in grafts at 4 and 8 weeks after implantation. None of the serotonin⁺ or GAD67⁺ neurons co-

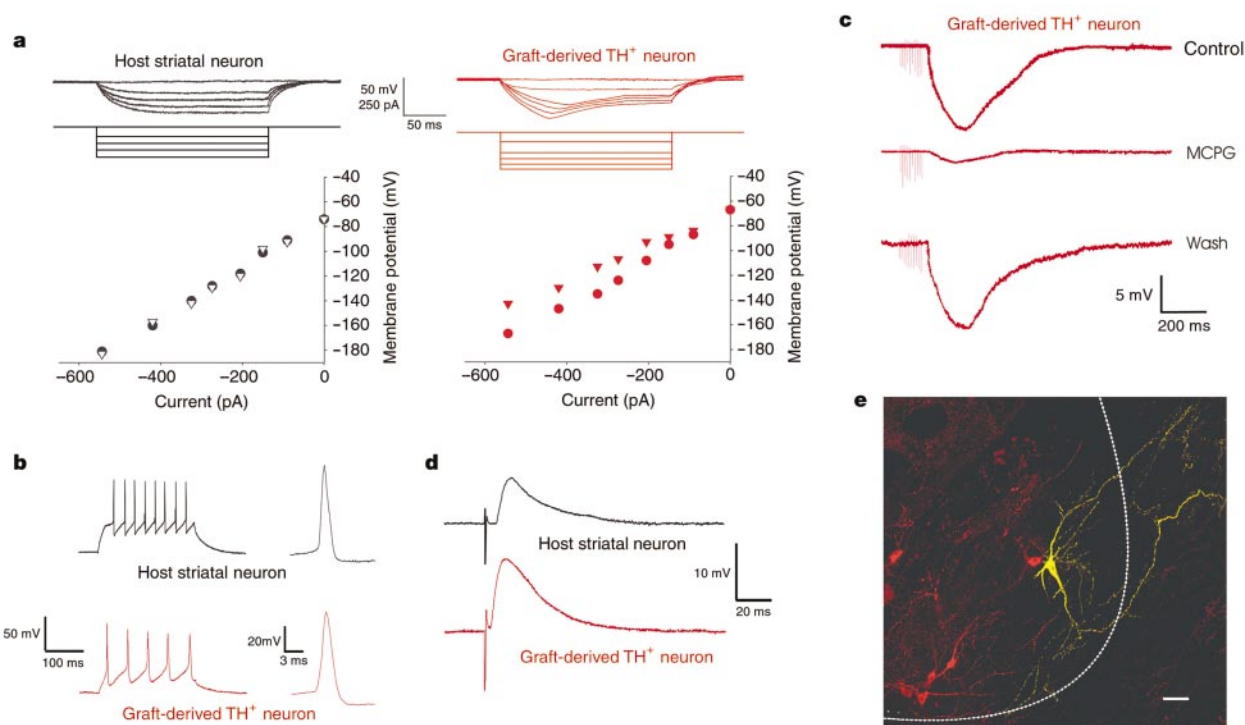


Figure 4 Electrophysiological properties of TH⁺ neurons. Simultaneous recordings were performed from neurons in the graft located on the graft–host border and neurons in the host striatum. **a**, Representative current–voltage relationship for a host striatal TH⁺ neuron and a TH⁺ neuron in the graft. The TH⁺ neurons display the time-dependent anomalous rectifier characteristic of dopamine-synthesizing cells after a hyperpolarizing pulse. Circles, the full extent of the immediate reduction in the membrane potential; triangles, the sustained membrane potential. **b**, Spike train profiles of a host striatal neuron and a graft-derived TH⁺ neuron. TH⁺ neurons fired broader action potentials at a

lower frequency than TH⁺ neurons. **c**, ES-derived TH⁺ neurons in the graft displayed a unique evoked IPSP mediated by activation of the metabotropic glutamate receptors. **d**, Extracellular stimulation in the centre of the graft resulted in an EPSP in both a graft-derived TH⁺ neuron and a host striatal neuron, indicating the presence of graft-to-host and graft-to-graft synapses. **e**, Confocal micrograph illustrating a biocytin-filled (green) TH⁺ neuron in the graft in close proximity to other non-filled, graft-derived TH⁺ neurons (red). The neuron was filled during recording. The filled neuron extends processes well into the host striatum. The dotted line shows the host–graft interface. Scale bar, 50 μm.

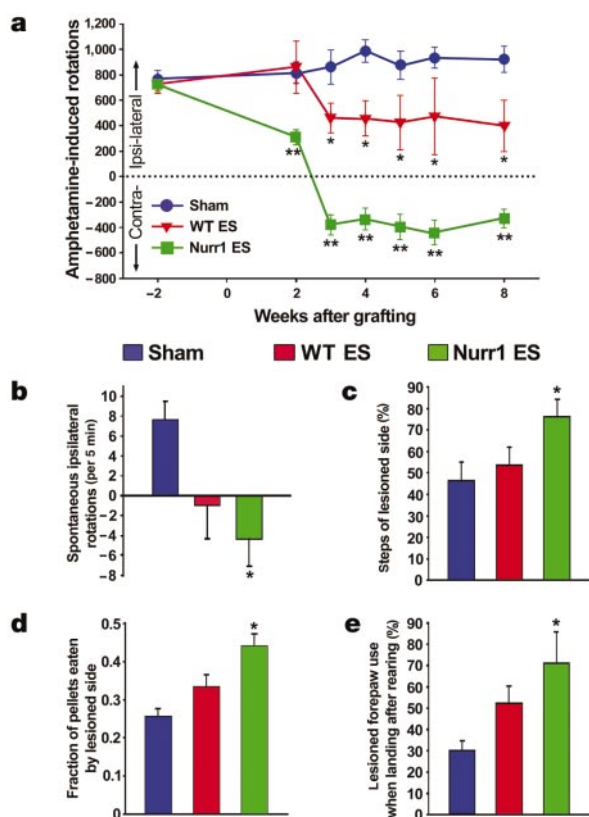


Figure 5 Behavioural effects of grafted Nurr1 ES cells. **a**, Analysis of amphetamine-stimulated rotations in animals grafted with neurons derived from wild-type (WT) ($n = 10$) or Nurr1 ES cells ($n = 15$) and sham controls ($n = 18$). **b–e**, Non-pharmacological evaluation of the animals grafted with either WT or Nurr1 ES cells (mean $\pm$ s.e.m.). **b**, Nine weeks after grafting and one week after the last injection of amphetamine, spontaneous turning behaviour was evaluated for 5 min. **c**, The results in the adjusting step test are expressed as a percentage of the lesioned side relative to the number of steps with the non-lesioned paw. **d**, In the paw-reaching test, the number of pellets eaten with the lesioned paw were normalized by the total number of pellets eaten during the 7-day test period. **e**, In the cylinder test, the use of each limb was measured when rearing and landing. The percentage of use of the lesioned-side limb relative to the total number of landings after rearing is expressed. Asterisk, $P < 0.05$; double asterisk, $P < 0.001$, compared with sham group.

expressed TH (data not shown). The great majority of neurons were TH⁺ and neuron number did not change significantly between 4 and 8 weeks (Fig. 3i). The stability of cell number in the graft is also an important issue because undifferentiated ES cells cause teratomas. The expression of Ki-67, a characteristic antigen found in dividing cells, was used to search for cell proliferation in the graft. No Ki-67⁺ cells were seen in the grafts (Fig. 3j) but they were abundant in sections of human glioma cells grafted into the adult rat brain (Fig. 3j, inset). Consistent with this finding, no teratomas were observed in animals that had received grafts of Nurr1 ES cells.

To test the electrophysiological properties of grafted neurons *in vivo*, rats were euthanized at several time points, up to 140 days after grafting, and brain slices (300 μ m thick, and containing the entire graft) were prepared for electrophysiological analysis. Using an infrared differential interference contrast (IR-DIC) microscope, neurons at the host-graft interface were preferentially targeted for recording. The properties of the recorded cells (Table 1) demonstrate that grafted TH⁺ cells display electrophysiological characteristics similar to those of mesencephalic neurons^{27,28}. For example, TH⁺ neurons subjected to hyperpolarizing voltage steps showed anomalous rectification *in vivo* (Fig. 4a). TH⁺ cells also showed

action potential frequency and duration properties distinct from TH⁺ neurons in the graft and in the parenchyma of the host striatum (Fig. 4b and Table 1). These differences are similar to previous reports comparing TH⁺ and TH⁺ mesencephalic neurons^{27,28}.

Mesencephalic dopamine neurons show a unique inhibitory postsynaptic potential (IPSP)²⁹. In five of six grafted TH⁺ neurons, a train of extracellular stimuli elicited this IPSP, which is dependent on metabotropic glutamate receptor type 1 (mGluR1) (Fig. 4c). In contrast, none of the five TH⁺ neurons showed this IPSP. Simultaneous recordings were performed from medium spiny neurons in the host striatum and from candidate TH⁺ neurons in the graft (Fig. 4d). Of 25 pairs of cells recorded, little or no spontaneous activity was observed and no direct synaptic connections were seen. However, when applying extracellular stimulation to a population of cells within the graft, excitatory postsynaptic potentials (EPSPs) were recorded in neurons both in the graft and in surrounding host cells up to 0.8 mm from the graft border (Fig. 4e). These results indicate that the ES-derived neurons develop functional synapses and show electrophysiological properties expected of mesencephalic neurons.

We next tested the behaviour of sham-operated animals and animals grafted with dopamine neurons differentiated to day 3 of stage 5. Amphetamine stimulation to animals lesioned unilaterally with 6-OH-DA induces a movement bias ipsilateral to the injection site. The sham group of animals showed a stable rotational bias (Fig. 5a). Animals grafted with 5×10^5 cells derived from WT ES cells showed a slight recovery in rotation behaviour. In contrast, the group grafted with Nurr1 ES cells showed a marked change in this parameter leading to consistent contralateral turning (Fig. 5a). The turning biases were preserved in sham grafted groups and groups grafted with Nurr1 ES cells when spontaneous rotations were measured (Fig. 5b). In addition, the recovery of these animals was evaluated with a battery of non-pharmacological tests that provide a more direct measure of motor deficits analogous to those found in human Parkinson's disease. We observed a significant improvement in the Nurr1 group in the adjusting step, cylinder and paw-reaching tests (Fig. 5c–e). Anatomical analysis showed that dopamine neurons were present in all the animals grafted with neurons derived from WT and Nurr1-expressing ES cells. Further studies are required to determine the relationship between the number of dopamine neurons and the behavioural response. However, the recovery observed in the behavioural tests shows that neurons derived from Nurr1 ES cells function in response to both pharmacological stimulation and also in spontaneous integrative tasks.

Discussion

The low efficiency of generation of dopamine neurons from primary cultures of fetal, neonatal cells or adult stem cells limits their therapeutic potential as donor cells. We report here that ES cells can efficiently generate midbrain precursors and dopamine neurons. The functional analysis of the ES-cell-derived neurons was conducted by anatomical, neurochemical, electrophysiological and behavioural tests. ES-cell-derived TH⁺ cells release dopamine, extend axons into the host striatum, form functional synaptic connections and modulate spontaneous and pharmacologically induced behaviours. Grafted fetal midbrain-specific dopamine neurons innervate the striatum and improve motor asymmetry^{1,30,31}. In contrast, hypothalamic dopamine neurons or nor-adrenaline neurons, which also express TH, did not innervate the striatum in a rat model of Parkinson's disease^{32–34}. Our data support the notion that ES-cell-derived neurons survive and function after grafting into the lesioned striatum.

Although the results presented here encourage the development of strategies involving neurons derived from ES cells in the treatment of neurological disease, further studies are needed both in rodent and primate systems to address the long-term safety and efficacy of these cells. Inappropriate cells may be included along

with the midbrain dopamine neurons. For example, tumour formation is a problem associated with ES cell grafting in models of Parkinson's disease³⁵. Under our conditions, differentiated neurons were enriched and dividing cells were not seen in a series of grafts that were analysed up to 8 weeks after transplantation; however, additional long-term data are needed to show that ES-derived cells do not divide *in vivo*. The dopamine and serotonin neurons of the mid- and hindbrain are also important in mood disorders and schizophrenia^{36,37}. Our results justify the continued study of the therapeutic benefits that can be derived from the use of ES cells, with the ultimate goal being the successful application of therapies based on ES cells in the treatment of neurological and psychiatric disease. □

Methods

Generation of Nurr1 ES cells and differentiation

A full-length cDNA fragment for rat Nurr1 was cloned into pCMV β vector (Clontech) by replacing β -galactosidase with a HA-tagged Nurr1 cDNA (HA-Nurr1) generated by fusing the HA tag (YPYDVPDYA) in frame at the 5' end to the Nurr1 cDNA. Mouse ES cells (R1) were co-transfected with pCMV-HA-Nurr1 and the neomycin resistance plasmid (pCDNA3.1; Invitrogen) by electroporation (0.25 kV, 500 μ F), and transfected clones were selected by growth in the presence of G418 (200 μ g ml⁻¹). The selected clones were screened for expression of HA-Nurr1 by immunoblot analysis, and five clones showing the highest levels of expression were chosen for differentiation experiments. Wild-type R1 ES cells were used as controls. To induce differentiation, ES cells were cultured as described⁸ or processed with Mouse Dopaminergic Neuron Differentiation Kit (R&D Systems) except for treatment with LIF at stage 2, during embryoid body (EB) formation.

Immunostaining of cultured cells and cryosections

Cultured cells were fixed in 4% paraformaldehyde in PBS. Perfused brain tissue was soaked in 20% sucrose overnight, frozen in isopentane cooled by solid CO₂ and cut on a cryostat at 35 μ m. The following antibodies were used: En-1, 1:50; M2, 1:100 (monoclonal, all from Developmental Studies Hybridoma Bank); Otx2 polyclonal³⁸, 1:1,000 (provided by G. Corte), Bf1 polyclonal³⁹, 1:1,000 (provided by E. Lai), Pax-2 polyclonal, 1:200 (Covance); TH polyclonal, 1:400 (Pelfreeze), or monoclonal, 1:1,000 (Sigma); serotonin polyclonal, 1:4,000 (Sigma); HA monoclonal, 1:200 (Santa Cruz Biotechnology); Nurr1 monoclonal, 1:1,000 (BD Transduction Laboratories); GAD67 polyclonal, 1:1,000 (Chemicon); and Ki-67 polyclonal, 1:200 (Novocastra). Appropriate fluorescence-tagged (Jackson ImmunoResearch Laboratories) or biotinylated (Vector Laboratories) secondary antibodies were used for visualization. Specimens were examined on Zeiss Axioplan or Zeiss LSM 510 confocal imaging system. Stacked optical sections were merged using Maximum Projection Software (Zeiss). Quantitative immunocytochemical data were expressed as means $\pm$ s.e.m.

Reverse-phase HPLC determinations of catecholamine

Catecholamine levels were determined after 15 days of differentiation. Dopamine release was stimulated in Hank's balanced salt solution (HBSS) containing 56 mM KCl (evoked release, 15 min incubation). Dopamine extraction and HPLC analysis have been described previously¹.

RT-PCR and immunoblot analysis

Total cellular RNA purification and RT-PCR was carried out as previously described⁸. The following primers were used to amplify target cDNA: Nurr1, 5'-TAAAGGCCGAGAGGTCGTC-3' and 5'-CTCTCTTGGGTTCCITGAGCC-3'; TH, 5'-CCTCTTGTCTC GGGCTGTAA-3' and 5'-CTGAGCTTGTCTTGGCGTCA-3'; Ptx3, 5'-AGGACGGCTC TGTGAAGA-3' and 5'-TTGACCGAGTTGAAGCGGAA-3'; En-1, 5'-TCAAGACTGAC TCACAGCAACCCC-3' and 5'-CTTTGTCCTGAACCGTGGTGGTAG-3'; c-RET, 5'-GCGCCCCGAGTGTGAGGAATGTGG-3' and 5'-GCTGATGAATGGGCGGCTT GTGC-3'; DAT, 5'-GGACCAATGCTTTCAGTGGTGGC-3' and 5'-GGATCCATGGGA GGTCCATGG-3'; AADC, 5'-CCTACTGGCTGCTCGACTAA-3' and 5'-GCGTACCAGTGACTCAAACTC-3'; Phox2a, 5'-TGGCGCTCAAGATGCACCTCA-3' and 5'-CGTTAGGTTGGGATTAGCGGT-3'; DBH, 5'-TTCCAATGTGCAGCTGAGTC-3' and 5'-GGTGCACTTGCTTGTGAGT-3'; GAPDH, 5'-ACCACAGTCCATGGCCAT CAC-3' and 5'-TCCACCACCTGTTGCTGTA-3'. Immunoblot analysis was performed as previously described³⁹.

Electrophysiology

Striatal slices (coronal, 300 μ m) were prepared and recordings were performed as previously reported⁴⁰. Briefly, grafted animals were euthanized at time points from 30 to 140 d after grafting, and slices were recorded in a submerged slice chamber at 30–32 °C, superfused at a rate of $\sim$ 2 ml min⁻¹ with artificial cerebrospinal fluid (ACSF). Extracellular stimulation was delivered through a bipolar stainless steel electrode. For recording of the mGluR1-mediated IPSPs, slices were bathed in ACSF containing picrotoxin (100 μ M), strychnine (1 μ M), eticlopride (100 nM), CNQX (25 μ M) and saclofen (10 μ M), as described²⁹.

Biocytin (0.2%) was added to the intracellular medium daily. Recordings were performed under visual guidance using an upright microscope. Voltage clamp recordings were performed at holding potentials of -60 to -70 mV, signals were

amplified using an Axopatch 200B amplifier, current clamp recordings were performed with a second amplifier (AxoClamp 2B), and all data were acquired and analysed on a PC running pClamp 8 (Axon Instruments). Drugs were applied by perfusion.

Transplantation and behavioural testing

Adult female Sprague–Dawley rats lesioned with 6-OH-DA were purchased (Taconic Farms). All surgical procedures were done according to guidelines of the National Institutes of Health (NIH) and National Institute of Neurological Disorders and Stroke (NINDS). WT and Nurr1 ES cells were trypsinized at day 3 of stage 5, and re-suspended at a density of 160,000 viable cells per μ l. Three microlitres of the cell suspension were grafted into the lesioned striatum of hemiparkinsonian rats (0.0 mm anteroposterior, +3.0 mm mediolateral and -5.0 mm dorsoventral of bregma and the meninges). In sham animals, 3 μ l of N2 medium was injected. The number of animals studied were: sham, 18; WT, 10; and Nurr1, 15. All animals were immunosuppressed with cyclosporine A (Neoral, Novartis, 10 mg kg⁻¹ d⁻¹, intraperitoneally, i.p.) starting 24 h before grafting. Amphetamine-induced (2.5 mg kg⁻¹, i.p.) rotational behaviour was evaluated for 70 min as described¹, before and after ES cell grafting, in animals with stable scores of >6 ipsilateral turns per min. To quantify neuronal survival after grafting, immuno-histochemical analysis was performed 4 and 8 weeks after grafting in the Nurr1 group. Several non-pharmacological tests were done as described, starting 9 weeks after grafting, as follows. (1) Spontaneous rotations³¹. (2) Adjusting step test⁴¹. Animals were moved in the backhand direction by a blind experimenter. The test was made three times per session, two sessions per day during three consecutive days. The results are expressed as a percentage of steps in lesioned compared with non-lesioned sides. Number of adjusting steps with the non-lesioned paw were: sham, 13.2 $\pm$ 0.3; WT, 13.0 $\pm$ 0.3; Nurr1, 12.6 $\pm$ 0.5. (3) Paw-reaching test. Animals were kept on a restricted diet until they lost 10–15% of their initial weight and then tested for 7 d in the appropriate chambers as described⁴². The results are given as the average fraction of pellets eaten by the lesioned side relative to the total number per day. In five naive animals, the fraction of pellets eaten by the right side was 0.51 $\pm$ 0.02. (4) Cylinder test⁴³. An observer, blind to the treatment, quantified two parameters: the number of wall contacts with each forelimb when rearing, and the number of times the rat used either forelimb for landing in at least 15 rearing-landing cycles. We expressed data as the percentage of use of the lesioned forepaw. For rearing, the percentages of contacts with the lesioned limb relative to the total number of contacts were not statically different between groups: sham, 4.4% $\pm$ 2.4; WT, 4.6% $\pm$ 5.2; Nurr1, 31.9% $\pm$ 20.0. For landing, the animals used almost always the non-lesioned paw either alone or together with the lesioned limb (sham and WT animals used the non-lesioned limb 100% of the time and Nurr1 used it 91.7 $\pm$ 8.3%). The significant increase ($P < 0.05$ relative to sham animals) in use of the lesioned side by the Nurr1 animals correlated with a significant decrease ($P < 0.05$) in the exclusive use of the non-lesioned paw. For these non-pharmacological tests, we studied five sham-operated, four WT and four Nurr1-grafted rats, in which the presence of dopamine neurons was also confirmed in the grafts. In all experiments, mean $\pm$ s.e.m. is represented. Statistical analysis was done by analysis of variance (ANOVA) followed by Fisher's test.

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1. Studer, L., Tabar, V. & McKay, R. D. Transplantation of expanded mesencephalic precursors leads to recovery in parkinsonian rats. *Nature Neurosci.* **1**, 290–295 (1998).
2. Sanchez-Pernaute, R., Studer, L., Bankiewicz, K. S., Major, E. O. & McKay, R. D. *In vitro* generation and transplantation of precursor-derived human dopamine neurons. *J. Neurosci. Res.* **65**, 284–288 (2001).
3. Thomson, J. A. *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147 (1998).
4. Shamblo, M. J. *et al.* Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc. Natl Acad. Sci. USA* **95**, 13726–13731 (1998).
5. McDonald, J. W. *et al.* Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. *Nature Med.* **5**, 1410–1412 (1999).
6. Rideout, W. M. III, Hochedlinger, K., Kyba, M., Daley, G. & Jaenisch, R. Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. *Cell* **109**, 17–27 (2002).
7. Kyba, M., Perlange, R. C. R. & Daley, G. Q. HoxB4 confers definitive lymphoid–myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell* **109**, 29–37 (2002).
8. Lee, S. H., Lumelsky, N., Auerbach, J. M. & McKay, R. D. Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. *Nature Biotechnol.* **18**, 675–679 (2000).
9. Okabe, S., Forsberg-Nilsson, K., Spiro, A. C., Segal, M. & McKay, R. D. Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells *in vitro*. *Mech. Dev.* **59**, 89–102 (1996).
10. Wallen, A. *et al.* Fate of mesencephalic AHD2-expressing dopamine progenitor cells in NURR1 mutant mice. *Exp. Cell Res.* **253**, 737–746 (1999).
11. Zetterstrom, R. H. *et al.* Dopamine neuron agenesis in Nurr1-deficient mice. *Science* **276**, 248–250 (1997).
12. Saucedo-Cardenas, O. *et al.* Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. *Proc. Natl Acad. Sci. USA* **95**, 4013–4018 (1998).
13. Le, W. *et al.* Selective agenesis of mesencephalic dopaminergic neurons in Nurr1-deficient mice. *Exp. Neurol.* **159**, 451–458 (1999).
14. Lumelsky, N. *et al.* Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science* **292**, 1389–1394 (2001).
15. Bader, A., Al-Dubai, H. & Weitzer, G. Leukemia inhibitory factor modulates cardiogenesis in embryoid bodies in opposite fashions. *Circ. Res.* **86**, 787–794 (2000).
16. Molne, M. *et al.* Early cortical precursors do not undergo LIF-mediated astrocytic differentiation. *J. Neurosci. Res.* **59**, 301–311 (2000).
17. Offield, M. F. *et al.* PDX-1 is required for pancreatic outgrowth and differentiation of the rostral

- duodenum. *Development* **122**, 983–995 (1996).
18. Hynes, M. & Rosenthal, A. Specification of dopaminergic and serotonergic neurons in the vertebrate CNS. *Curr. Opin. Neurobiol.* **9**, 26–36 (1999).
19. Wurst, W. & Bally-Cuif, L. Neural plate patterning: upstream and downstream of the isthmus organizer. *Nature Rev. Neurosci.* **2**, 99–108 (2001).
20. Ye, W., Shimamura, K., Rubenstein, J. L., Hynes, M. A. & Rosenthal, A. FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell* **93**, 755–766 (1998).
21. Broccoli, V., Boncinelli, E. & Wurst, W. The caudal limit of Otx2 expression positions the isthmus organizer. *Nature* **401**, 164–168 (1999).
22. Joyner, A. L., Liu, A. & Millet, S. Otx2, Gbx2 and Fgf8 interact to position and maintain a mid-hindbrain organizer. *Curr. Opin. Cell Biol.* **12**, 736–741 (2000).
23. Tao, W. & Lai, E. Telencephalon-restricted expression of BF-1, a new member of the HNF-3/fork head gene family, in the developing rat brain. *Neuron* **8**, 957–966 (1992).
24. Wurst, W., Auerbach, A. B. & Joyner, A. L. Multiple developmental defects in Engrailed-1 mutant mice: an early mid-hindbrain deletion and patterning defects in forelimbs and sternum. *Development* **120**, 2065–2075 (1994).
25. Smidt, M. P. *et al.* A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b. *Nature Neurosci.* **3**, 337–341 (2000).
26. Gerfen, C. R., Baimbridge, K. G. & Thibault, J. The neostriatal mosaic: III. Biochemical and developmental dissociation of patch-matrix mesostriatal systems. *J. Neurosci.* **7**, 3935–3944 (1987).
27. Reppert, S. *et al.* Identified postnatal mesolimbic dopamine neurons in culture; morphology and electrophysiology. *J. Neurosci.* **12**, 4264–4280 (1992).
28. Rohrbacher, J., Ichinohe, N. & Kitai, S. T. Electrophysiological characteristics of substantia nigra neurons in organotypic cultures: spontaneous and evoked activities. *Neuroscience* **97**, 703–714 (2000).
29. Fiorillo, C. D. & Williams, J. T. Glutamate mediates an inhibitory postsynaptic potential in dopamine neurons. *Nature* **394**, 78–82 (1998).
30. Abrous, D. N., Shaltot, A. R., Torres, E. M. & Dunnett, S. B. Dopamine-rich grafts in the neostriatum and/or nucleus accumbens: effects on drug-induced behaviours and skilled paw-reaching. *Neuroscience* **53**, 187–197 (1993).
31. Nikkhah, G., Duan, W. M., Knappe, U., Jödicke, A. & Björklund, A. Restoration of complex sensorimotor behaviour and skilled forelimb use by a modified nigral cell suspension transplantation approach in the rat Parkinson model. *Neuroscience* **56**, 33–43 (1993).
32. Zuddas, A., Corsini, G. U., Barker, J. L., Kopin, I. J. & di Porzio, U. Specific reinnervation of lesioned mouse striatum by grafted mesencephalic dopaminergic neurons. *Eur. J. Neurosci.* **3**, 77–85 (1991).
33. Hudson, J. L., Bickford, P., Johansson, M., Hoffer, B. J. & Stromberg, I. Target and neurotransmitter specificity of fetal central nervous system transplants: importance for functional reinnervation. *J. Neurosci.* **14**, 283–290 (1994).
34. Björklund, A. & Lindvall, O. Cell replacement therapies for central nervous system disorders. *Nature Neurosci.* **3**, 537–544 (2000).
35. Björklund, L. M. *et al.* Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. *Proc. Natl Acad. Sci. USA* **99**, 2344–2349 (2002).
36. Gross, C. *et al.* Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. *Nature* **416**, 396–400 (2002).
37. Sawa, A. & Snyder, S. H. Schizophrenia: diverse approaches to a complex disease. *Science* **296**, 692–695 (2002).
38. Mallamaci, A., Di Blas, E., Briata, P., Boncinelli, E. & Corte, G. OTX2 homeoprotein in the developing central nervous system and migratory cells of the olfactory area. *Mech. Dev.* **58**, 165–178 (1996).
39. Le, W. L., Conneely, O. M., He, Y., Jankovic, J. & Appel, S. H. Reduced Nurr1 expression increases the vulnerability of mesencephalic dopamine neurons to MPTP-induced injury. *J. Neurochem.* **73**, 2218–2221 (1999).
40. Auerbach, J. M., Eiden, M. V. & McKay, R. D. Transplanted CNS stem cells form functional synapses *in vivo*. *Eur. J. Neurosci.* **12**, 1696–1704 (2000).
41. Olsson, M., Nikkhah, G., Bentlage, C. & Björklund, A. Forelimb akinesia in the rat Parkinson model: Differential effects of dopamine agonists and nigral transplants as assessed by a new stepping test. *J. Neurosci.* **15**, 3863–3875 (1995).
42. Montoya, C. P., Campbell, H. L., Pemberton, K. D. & Dunnett, S. B. The ‘staircase test’: a measure of independent forelimb reaching and grasping abilities in rats. *J. Neurosci. Methods* **36**, 219–228 (1991).
43. Schallert, T., Fleming, S. M., Leasure, J. L., Tillerson, J. L. & Bland, S. T. CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. *Neuropharmacology* **39**, 777–787 (2000).

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CO and H_3^+ in the protoplanetary disk around the star HD141569

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Massive planets have now been found orbiting about 80 stars. A long outstanding question critical to theories of planet formation has been the timescale on which gas-giant planets form; in particular, stars more massive than the Sun may blow away the surrounding gas associated with their formation more quickly than it can be accumulated by the protoplanetary cores¹. Evidence for a protoplanet around a Herbig AeBe star (such stars are 2–3 times more massive than the Sun) would constrain the timescale of planet formation. Here we report the detection of CO and H_3^+ emission from the 5–10-million-year-old Herbig AeBe star HD141569. We interpret the CO data as indicating that the inner disk surrounding the star is past the early phase of accretion and planetesimal formation, and that most of the gas has been cleared out to a distance of more than 17 astronomical units. CO effectively destroys H_3^+ (ref. 2), so their presence in the same source is surprising. Moreover, H_3^+ line emission has previously been detected only from the atmospheres of the giant planets in the Solar System^{3,4}. The H_3^+ and CO may therefore be distributed in the disk at different circumstellar distances, or, alternatively, H_3^+ may be located in the extended envelope of a protoplanet.

The Herbig AeBe (HAeBe) star, HD141569, is a nearby (99 parsecs, pc) B9.5Ve pre-main-sequence star with an extended disk (radius, $R = 400$ astronomical units, AU) that has been shown to have a gap at 250 AU. This star is particularly interesting because it is a transitional object passing from the pre-main-sequence HAeBe stage onto the zero-age main sequence^{5–7}. CO has been detected with submillimetre observations in the colder disk region (60 K) inward of 130 AU (ref. 8); submillimetre observations are not sensitive to warm gas in the inner regions. The inner portion of the disk (<10 AU) has little or no dust⁹; most of the gas and dust in the inner disk of a star of this age has been radiatively cleared or accreted into larger grains and planetesimals. There is evidence of dust grains (smaller than $2\ \mu\text{m}$) from 10–100 AU, but dust of this size has a short disk lifetime as it is effectively removed by stellar radiation effects⁵. These mid-infrared-emitting grains are not primordial and are probably replenished through collisions of larger bodies on a timescale of hundreds to thousands of years^{5,6,10}. High-resolution imaging from the Hubble Space Telescope (HST) has not been able to ascertain the detailed structure in the inner disk⁶.

Emission spectra of CO and H_3^+ presented in Fig. 1 and Fig. 2 were obtained with the NASA Infrared Telescope (IRTF) using the cryogenic echelle spectrograph (CSHELL)¹¹. Assuming the molecular material is distributed uniformly around the star in the form of a disk, the linewidth of CO (Fig. 1) constrains the deprojected orbital velocity to $11 \pm 3\ \text{km s}^{-1}$ (HD141569 is tilted from the plane of the sky by approximately $39 \pm 3^\circ$ (ref. 6)). This velocity places an inner limit on the location of CO at 17 AU where stellar radiation continues to dissociate the remaining molecules and push the denser disk regions outward. The line-by-line profiles of the CO emission lines follow the point-spread function of the continuum, indicating that it is not an extended source within our resolution. The outer detection limit of CO is constrained to less than 50 AU. This value derives from the measured 0.3 arcsec half-width of the line profile that corresponds to a disk radius of 30 AU at 99 pc for HD141569. When the disk is deprojected to zero inclination, the

outer extent for the CO gas is determined to be 50 AU. In contrast to the outer regions probed by submillimetre measurements¹², these near-infrared observations sample the inner protoplanetary disk. An analysis of the line strengths provides a measure of the CO column density and a mass of $10^{19}\ \text{g}$ ($10^{-8} M_{\text{earth}}$, where M_{earth} is the mass of the Earth) for CO gas that remains in the $17 < R < 50$ AU region around HD141569.

Planet formation has been known for many years to be tied to the accretion and evolution of gas and dust in disks around young stars. Although the timescales are not well-constrained, the solid terrestrial-like cores needed to form giant planets develop in approximately 10^6 years, whereas massive gas envelopes require about 10^7 years^{13–15}. The data indicate that the inner disk (<50 AU) of the 5–10-million-year-old HD141569 system has been largely cleared of CO. There is not enough gas in the disk to support the formation of a giant planet, even if the nominal $(\text{CO})/(\text{H}_2) \approx 10^{-4}$ ratio¹² is increased by several orders of magnitude. It is possible, however, that a jovian-type protoplanet has previously evolved from the disk.

Figure 2a and b presents the observations of two emission lines of H_3^+ from HD141569. The telluric absorption lines in Fig. 2 were used to calibrate the wavelength dispersion in the spectra and provide the observed positions (9 August 2001) of $2,529.61 \pm 0.03\ \text{cm}^{-1}$ for Q(1,0) and $2,508.95 \pm 0.03\ \text{cm}^{-1}$ for Q(3,0). (See Fig. 2 legend for details of line nomenclature.) The single pixel dispersion, $\sim 0.03\ \text{cm}^{-1}$ ($3\ \text{km s}^{-1}$), is the primary source of the uncertainty in all measurements. The laboratory position of the Q(1,0) line is $2,529.72\ \text{cm}^{-1}$ and Q(3,0) is $2,509.08\ \text{cm}^{-1}$. The differences in the observed positions compared to the laboratory positions are a result of the geocentric radial velocity contribution of $+27\ \text{km s}^{-1}$ (9 August 2001) and the stellar/heliocentric component of $-8 \pm 3\ \text{km s}^{-1}$. The heliocentric velocity was derived from our CO measurements and is consistent with submillimetre measurements⁸. Similarly, the heliocentric velocity of the two H_3^+ lines is determined to be $-13 \pm 4\ \text{km s}^{-1}$ (or $0.11\ \text{cm}^{-1}$ relative to the rest position). The two lines of H_3^+ are displaced by $-5 \pm 5\ \text{km s}^{-1}$ relative to CO. In this wavelength region, there are no significant molecular or atomic transitions that are likely to be confused with H_3^+ .

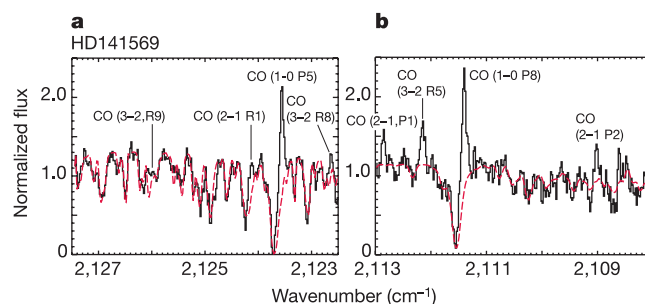


Figure 1 The CO spectra of HD141569. **a, b**, The CO observations were obtained with CSHELL at IRTF on 6, 7, and 9 August 2001 at a resolving power $\sim 21,500$. The night-sky telluric lines are modelled (red dashed line) with the Spectrum Synthesis Program²⁴ accessing the '92HITRAN molecular database²⁵ and are used to establish the wavelength calibration. The atmospheric seeing conditions were $\sim 0.6''$ (5, 6 and 9 August). The observed CO line strengths from the $\nu = 2 \rightarrow 1$ and $\nu = 3 \rightarrow 2$ transitions are about half the intensity of the $\nu = 1 \rightarrow 0$ transitions and indicate the CO is pumped by strong stellar ultraviolet radiation from the B9.5e star. The line intensities for six lines from the $\nu' = 1$ band of CO provide a rotational temperature of $190 \pm 30\ \text{K}$. The flux of the continuum is $2.0 \times 10^{-15}\ \text{erg s}^{-1}\ \text{cm}^{-2}\ \text{per cm}^{-1}$ (or $4.2 \times 10^{-17}\ \text{erg s}^{-1}\ \text{cm}^{-2}$ per pixel). Using the $\nu = 1 \rightarrow 0$, $\nu = 2 \rightarrow 1$, and $\nu = 3 \rightarrow 2$ vibrational bands, the column density of the detected CO gas is of the order of $10^{11}\ \text{cm}^{-2}$. The heliocentric velocity of the CO surrounding HD141569 is $-8 \pm 3\ \text{km s}^{-1}$. The heliocentric velocity measurement is consistent at 1σ with that reported for HD141569 elsewhere^{8,26,27}.

The only previously detected H_3^+ emission lines originate from the dense upper atmospheres of the Solar System giant planets, where the strongest source is Jupiter^{3,4}. In this case, the production of H_3^+ in the auroral regions is instigated by the ionization of neutral H_2 by electron collision. The ionized H_2 quickly combines with another H_2 to form H_3^+ (that is, $\text{H}_2 + \text{H}_2^+ \rightarrow \text{H}_3^+ + \text{H}$) because the proton affinity of H_2 is greater than that of hydrogen. In the jovian atmosphere, the upper energy levels of H_3^+ are populated by thermal excitation as well as non-thermal mechanisms such as resonant exchange of vibrational energy with H_2 , resulting in a ro-vibrational temperature of 1,100 K (refs 4, 16, 17). For Uranus, 740 K is measured¹⁸. Unlike the planetary atmospheres, the R(1,0) line at $2,725.90 \text{ cm}^{-1}$ was not observed in HD141569, which suggests that the excitation mechanism is different from that of Jupiter. Further efforts will be required to test infrared or various other pumping processes as potential excitation mechanisms of H_3^+ in HD141569. H_3^+ is found in absorption in a variety of astronomical sources, including dense and diffuse molecular clouds^{19,20} where cosmic ray irradiation leads to the formation of the diatomic ion H_2^+ that reacts exothermically with H_2 to produce H_3^+ (ref. 2).

Although the excitation mechanism of H_3^+ is not clear for HD141569, there are two possibilities for the source of the observed emission: the inner edge of the circumstellar disk or the upper atmosphere of a gas giant protoplanet. The inner edge of the

circumstellar disk (Fig. 3) can potentially form H_3^+ , as ultraviolet radiation from the central star ionizes H_2 in this region. The resulting H_2^+ will rapidly react with H_2 to form H_3^+ . If HD141569 is a typical HAeBe disk system we should expect HAeBe disks to show various amounts of H_3^+ . As an example, we present spectra for the HAeBe AB Aurigae (Fig. 2c, d). The more massive disk might be expected to produce H_3^+ in larger quantities; however, it is not detected.

In the case where H_3^+ is assumed to be collisionally excited, the critical density of H_2 necessary for H_3^+ emission is approximately 10^{11} cm^{-3} (ref. 2). Using the estimated value of the amount of H_2 in the disk around HD141569 ($400 M_{\text{earth}}$) (ref. 8) suggests that collisionally excited H_3^+ cannot be distributed uniformly in the disk because the entire amount of H_2 would have to be contained in a volume of $\sim 1 \text{ AU}^3$. Any reaction with CO efficiently destroys the H_3^+ molecule², so it may be natural to suspect that the two molecules originate from different locations. In addition, the measured line positions of H_3^+ hint at a slight blue shift ($5 \pm 5 \text{ km s}^{-1}$) that might suggest separate locations. Thus the second possible source of H_3^+ might result from the atmosphere of an extended gas protoplanet (see Fig. 3).

Giant planet formation is closely related to disk formation, where the timescale for growth and evolution of protoplanets is determined by the rate of gas and dust accretion and dispersal. As a circumstellar disk evolves, it may become clumpy and form a gas protoplanet^{21–23} as described by either the core accretion or disk instability model. This leads to the possibility that jovian quantities of H_2 (and subsequently H_3^+) remain in a region interior to the current star–disk inner boundary, perhaps in the extended environment of a gas giant planet before hydrodynamic collapse to a Jupiter-sized planet (see Fig. 3). A massive protoplanet five times the mass of Jupiter can be upwards of 2 AU in diameter as defined by a Hill sphere at 7 AU. The effective area is $\sim 3,000$ times smaller than the beam size used in the observations. The H_3^+ column density for a $100 \text{ AU} \times 100 \text{ AU}$ region (from a $1'' \times 1''$ beam at 99 pc) is 10^{11} cm^{-2} at a temperature of 420 K. Assuming the source is a 2-AU diameter protoplanet (see Fig. 3) the resulting column density is $\sim 10^{14} \text{ cm}^{-2}$. The column density of H_3^+ for Jupiter has been reported to be of the order of 10^{11} cm^{-2} (ref. 16) and $\sim 10^{12}–10^{13} \text{ cm}^{-2}$ (ref. 17). The

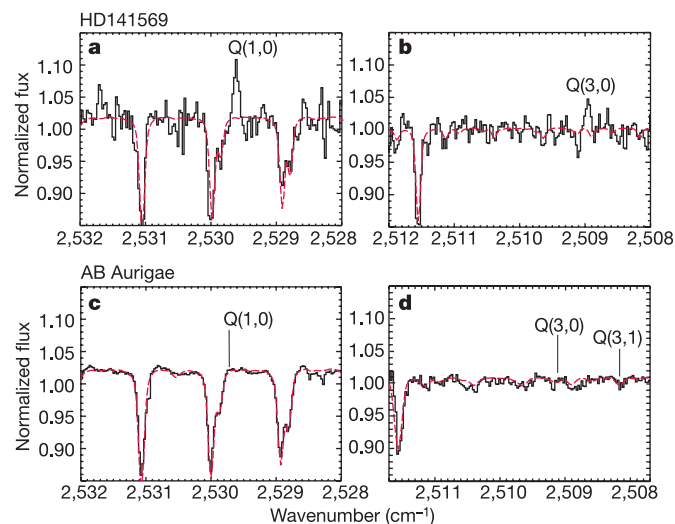


Figure 2 Detections and non-detections of H_3^+ emission. The spectra for HD141569 (a, b) showing the H_3^+ detection and a similar spectral region for AB Aurigae (c, d) showing a non-detection. The deep absorption features are atmospheric N_2O that are fitted with the Spectrum Synthesis Program model (red dashed lines) to calibrate the frequency scale of the spectrum. The observations were obtained using CSHELL on IRTF on 9 August 2001 and 3 February 2002. The Q(1,0) line at $2,529.61 \pm 0.03 \text{ cm}^{-1}$ (9 August 2001) is detected at 5σ ($1.7 \times 10^{-5} \text{ erg s}^{-1} \text{ cm}^{-2} \text{ sr}^{-1}$). The Q(3,0) line at $2,508.95 \pm 0.03 \text{ cm}^{-1}$ is detected at 3σ ($1.1 \times 10^{-5} \text{ erg s}^{-1} \text{ cm}^{-2} \text{ sr}^{-1}$). The flux of the continuum is $2.2 \times 10^{-15} \text{ erg s}^{-1} \text{ cm}^{-2} \text{ per cm}^{-1}$. To produce the signal-to-noise ratio presented for the Q(3,0) line, the 3 February 2002 observations were corrected for the velocity difference resulting from Earth's motion and added to the 9 August 2001 observations. The upper limit for the Q(3,1) line at $2,508.17 \text{ cm}^{-1}$ is $< 3.9 \times 10^{-6} \text{ erg s}^{-1} \text{ cm}^{-2} \text{ sr}^{-1}$. A thorough search provided no atomic or molecular transitions consistent with the line positions of Q(1,0) and Q(3,0). The only identified atomic lines in the Q(1,0) region are C I ($2,529.61 \text{ cm}^{-1}$ 3P–3Po) and Si I ($2,529.904 \text{ cm}^{-1}$ 3D_{5/2}–3/2[3/2]); these weak transitions are not consistent with the observed line and no other C I or Si I lines with similar lower state energies are detected in this region. (H_3^+ lines are generally denoted by the quantum numbers of the lower level of the transition: J and G . Thus the lines of H_3^+ dealt with in this Letter are described by [P]Q[R](J,G) where P, Q and R represent $\Delta J = -1, 0$ and $+1$ respectively.)

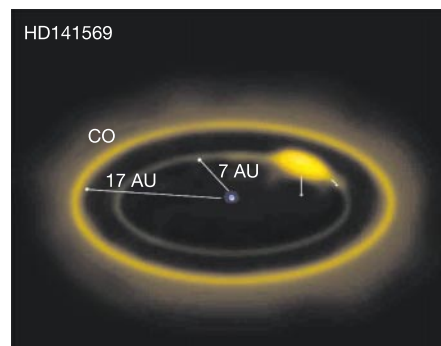


Figure 3 Speculative illustration showing the inner edge of the CO gas disk and a gas giant protoplanet slightly closer to the central star from which the H_3^+ may originate. The illustration suggests that the H_3^+ emission, if from an extended protoplanetary envelope, has a unique velocity with respect to CO. In this case, the resultant Doppler velocity $-13 \pm 4 \text{ km s}^{-1}$ for the two H_3^+ lines relative to CO at $-8 \pm 3 \text{ km s}^{-1}$ hints at a slightly blue-shifted source of H_3^+ but additional high-resolution observations will be needed to investigate the scenario. The dust component of the inner disk region of HD141569 (refs 5, 9) may result from the numerous planetesimal collisions expected as the nebular envelope evolves to a planetary atmosphere. The CO in this protoplanet envelope is unlikely to be a factor in destroying H_3^+ as differentiation of the heavier molecules and dust will leave the extended atmosphere dominated by H_2 .

presence of a less evolved inner disk around AB Aur⁹ suggests it may not yet have evolved to the gas-accretion phase of giant planet formation. Even if planet formation has begun around AB Aur, the observations presented in Fig. 2 are not sensitive enough to detect a potential protoplanet owing to the increased stochastic noise. Infrared spectroscopic studies of CO and H₃⁺ provide a technique to study the protoplanetary disk regions that will provide a better understanding of the early disk accretion and clearing processes, as well as the chemical processing that occurs before planet formation. In addition, H₃⁺ has the potential to provide a new search technique for planets in the very early stages of development. □

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1. Natta, A., Grinin, V. & Mannings, V. in *Protostars and Planets IV* (eds Mannings, V., Boss, A. P. & Russell, S. S.) 559–588 (Univ. Arizona Press, Tucson, 2000).
2. Oka, T. A search for interstellar H₃⁺. *Phil. Trans. R. Soc. Lond. A* **303**, 543–549 (1981).
3. Maillard, J. P., Drossart, P., Watson, J. K. G., Kim, S. J. & Caldwell, J. H₃⁺ fundamental band in Jupiter's auroral zones at high resolution from 2400 to 2900 cm⁻¹. *Astrophys. J.* **363**, L37–L41 (1990).
4. Drossart, P. *et al.* Detection of H₃⁺ on Jupiter. *Nature* **340**, 539–541 (1989).
5. Fisher, R. S., Telesco, C. M., Piña, R. K., Knacke, R. F. & Wyatt, M. C. Detection of extended thermal infrared emission around the Vega-like source HD 141569. *Astrophys. J.* **532**, L141–L144 (2000).
6. Weinberger, A. J. *et al.* The circumstellar disk of HD141569 imaged with NICMOS. *Astrophys. J.* **525**, L53–L56 (1999).
7. van den Ancker, M. E., de Winter, D. & Tjin A Dije, H. R. HIPPARCOS photometry of Herbig Ae/Be stars. *Astron. Astrophys.* **330**, 145–154 (1998).
8. Zuckerman, B., Forveille, T. & Kastner, J. H. Inhibition of giant planet formation by rapid gas depletion around young stars. *Nature* **373**, 494–496 (1995).
9. Malfait, K., Bogaert, E. & Waelkens, C. An ultraviolet, optical and infrared study of Herbig Ae/Be stars. *Astron. Astrophys.* **331**, 211–223 (1998).
10. Weinberger, A. J., Rich, R. M., Becklin, E. E., Zuckerman, B. & Matthews, K. Stellar companions and the age of HD 141569 and its circumstellar disk. *Astrophys. J.* **544**, 937–943 (2000).
11. Tokunaga, A. T., Toomey, D. W., Carr, J., Hall, D. N. B. & Epps, H. W. *Proc. Instrumentation in Astronomy VII* 131–143 (Society of Photo-Optical Instrumentation Engineers, Bellingham, WA, 1990).
12. Thi, W. F. *et al.* H₂ and CO emission from disks around T Tauri and Herbig Ae pre-main-sequence stars and from debris disks around young stars: warm and cold circumstellar gas. *Astrophys. J.* **561**, 1074–1094 (2001).
13. Ruden, S. P. & Pollack, J. B. The dynamical evolution of the protosolar nebula. *Astrophys. J.* **375**, 740–760 (1991).
14. Lissauer, J. J. Planet formation. *Annu. Rev. Astron. Astrophys.* **31**, 129–174 (1993).
15. Podolak, M., Hubbard, W. B. & Pollack, J. B. in *Protostars and Planets III* (ed. Levy, E. H. Lunine, J. I.) 1109–1147 (Univ. Arizona Press, Tucson, 1993).
16. Miller, S., Tennyson, J. & Joseph, R. D. Infrared emission of H₃⁺ in the atmosphere of Jupiter in the 2.1 and 4.0 μm region. *Astrophys. J.* **360**, L55–L58 (1990).
17. Oka, T. & Geballe, T. R. Observations of the 4 μm fundamental band of H₃⁺ in Jupiter. *Astrophys. J.* **351**, L53–L56 (1990).
18. Trafton, L. M., Geballe, T. R., Miller, S., Tennyson, J. & Ballester, G. E. Detection of H₃⁺ from Uranus. *Astrophys. J.* **405**, 761–766 (1993).
19. McCall, B. J., Geballe, T. R., Hinkle, K. H. & Oka, T. Observations of H₃⁺ in dense molecular clouds. *Astrophys. J.* **522**, 338–348 (1999).
20. McCall, B. J., Hinkle, K. H., Geballe, T. R. & Oka, T. H₃⁺ in dense and diffuse clouds. *Faraday Discuss.* **109**, 267–280 (1998).
21. Wuchterl, G., Gillot, T. & Lissauer, J. J. in *Protostars and Planets IV* (ed. Mannings, V. Boss, A. P. Russell, S. S.) 1081–1110 (Univ. Arizona Press, Tucson, 2000).
22. Boss, A. P. *Planetary Systems in the Universe* 202–205 (International Astronomical Union, IAU Symp. 202, Manchester, UK, 2000).
23. Boss, A. P. Formation of extrasolar giant planets: core accretion or disk instability? *Earth Moon Planets* **81**, 19–26 (1998).
24. Kunde, V. G. & Maguire, W. C. A Direct Integration Transmittance Model. *J. Quant. Spectrosc. Radiat. Transfer* **14**, 803–817 (1974).
25. Rothman, L. S. *et al.* The HITRAN molecular database: editions of 1991 and 1992. *J. Quant. Spectrosc. Radiat. Transfer* **48**, 469–507 (1992).
26. Dunkin, S. D., Barlow, M. J. & Ryan, S. G. High-resolution Spectroscopy of Vega-like Stars—I. Effective Temperatures, Gravities and Photospheric Abundances. *Mon. Not. R. Astron. Soc.* **286**, 604–616 (1997).
27. Frisch, P. C. Radial velocities in selected B-G stars. *Astrophys. J. Suppl.* **65**, 313–317 (1987).

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Interpretation of tomography and spectroscopy as dual forms of quantum computation

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It is important to be able to determine the state of a quantum system and to measure properties of its evolution. State determination can be achieved using tomography¹, in which the system is subjected to a series of experiments, whereas spectroscopy can be used to probe the energy spectrum associated with the system's evolution. Here we show that, for a quantum system whose state or evolution can be modelled on a quantum computer, tomography and spectroscopy can be interpreted as dual forms of quantum computation². Specifically, we find that the phase estimation algorithm³ (which underlies a quantum computer's ability to perform efficient simulations⁴ and to factorize large numbers⁵) can be adapted for tomography or spectroscopy. This is analogous to the situation encountered in scattering experiments, in which it is possible to obtain information about both the state of the scatterer and its interactions. We provide an experimental demonstration of the tomographic application by performing a measurement of the Wigner function (a phase space distribution) of a quantum system. For this purpose, we use three qubits formed from spin-1/2 nuclei in a quantum computation involving liquid-state nuclear magnetic resonance.

Quantum computation is best known for its potential ability to efficiently solve otherwise difficult problems like factoring large numbers⁵. The technology needed to realize this ability is still elusive. For now, the ideas of quantum computation can be used for illuminating fundamental processes and methods in quantum mechanics. Here we show that tomography and spectroscopy are both aspects of the same quantum computation that can be represented by a 'scattering' circuit. Versions of this circuit also play a crucial role⁶ in many of the quantum algorithms exhibiting marked improvements over the best classical counterparts. Typically, when analysing a quantum system one is interested in the evolution of an initial state over a given time period. This is implemented on a quantum computer by decomposing the evolution operator for the time period into a sequence of elementary operations called quantum gates⁷ (a combination of elementary gates forms a quantum 'circuit'). The fact that most reasonable quantum evolutions can be decomposed in this way is an observation that was suggested by Feynman⁸ and established more formally by Lloyd⁴. The circuit representing an evolution can be readily modified to account for experimental conditions under our control, or to include interactions with additional degrees of freedom used as probes.

In an experimental setting, tomography is used to determine the state of a quantum system, whereas spectroscopy is required for obtaining information about the energy spectrum of the system. The natural setting for our work involves a quantum system whose

state or evolution can be modelled on a quantum computer. To make the connection between tomography and spectroscopy, we introduce the family of quantum algorithms² represented by the ‘scattering’ circuit of Fig. 1. Versions of this circuit play an important part in many quantum algorithms. For pure input states, it occurs in Kitaev’s solution to the Abelian stabilizer problem³. This was adapted by Cleve *et al.*⁶ to revisit most quantum algorithms. Abrams and Lloyd⁹ gave another presentation of the algorithm as a tool for finding random, approximate eigenvalues of certain hamiltonians. The simple extension to mixed states is described in ref. 10.

Given sufficiently many independent instances of the experiment required to implement the scattering circuit, the measurements yield the expectation values $\langle\sigma_z\rangle$ and $\langle\sigma_y\rangle$ of the Pauli spin operators σ_z and σ_y for the ancilla qubit used as a probe particle. The expectation values obtained by use of the scattering circuit have the following property:

$$\langle\sigma_z\rangle = \text{Re}[\text{Tr}(U\rho)], \quad \langle\sigma_y\rangle = \text{Im}[\text{Tr}(U\rho)] \quad (1)$$

The state ρ and the operator U appear symmetrically in the right-hand sides of equation (1). As a result, the scattering circuit can be used to measure properties of U or ρ by using known instances of ρ or U , respectively. Two applications are spectroscopy and tomography. In spectroscopy we wish to determine properties of the spectrum of an evolution U . We show below how this can be done with the scattering circuit by preparing suitable initial states ρ . The purpose of tomography is to determine the unknown but preparable state ρ . The scattering circuit can be used to accomplish this by using a basis of operators U . Because the same circuit can be used either as a spectrometer or as a ‘tomographer’, these can now be viewed as dual forms of quantum computation.

We first discuss the use of the scattering circuit as a spectrometer. We consider two possible applications. The first is to determine spectral properties of the evolution $U = \exp(-iht)$ realized by a quantum physics simulation on a quantum computer, where h is the hamiltonian and t is the time. The second is to benchmark an accessible quantum device inducing U and determine features of the eigenvalue distribution of U . In both cases it is necessary to be able to coherently realize U conditional on the internal state of the ancilla qubit. There are standard techniques for doing this⁷ if U is realized as a quantum computation. The simplest spectroscopy application involves preparing the system in a completely mixed initial state $\rho = I/N$, where $N = 2^n$ is the dimensionality of the n -qubit state space of the system, where I is the identity operator. The final

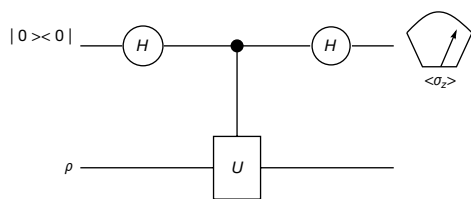


Figure 1 The scattering circuit. The circuit represents a sequence of instructions for applying operations to quantum systems. The horizontal lines represent the time-lines of the quantum systems of interest. The operations are applied in left-to-right order. In this case, there are two systems. The bottom system models the physical system of interest, and is initially in the state ρ (a density matrix). The top system is an ancilla qubit acting as a ‘probe particle’. It is initialized in the state $|0\rangle$, whose density matrix is $|0\rangle\langle 0|$. The qubit can be thought of as a spin-1/2 particle with 0 and 1 representing the ‘down’ and ‘up’ states, respectively. The circuit consists of the following steps: (1) apply a Hadamard transform H to the ancilla qubit. Because $H|0\rangle = (|0\rangle + |1\rangle)/\sqrt{2}$, $H|1\rangle = (|0\rangle - |1\rangle)/\sqrt{2}$, the new state of the qubit is $(|0\rangle + |1\rangle)/\sqrt{2}$. (2) Apply a ‘controlled- U ’ operator, which does nothing if the state of the ancilla is $|0\rangle$ and applies U to the system if the ancilla is in state $|1\rangle$. (3) Apply another Hadamard gate to the ancilla and perform measurements of its spin polarizations along the z and y axes. The polarization along the z axis ($\langle\sigma_z\rangle$) yields $\text{Re}[\text{Tr}(\rho U)]$ for any unitary operator U that can be controlled.

measurement yields $\langle\sigma_z\rangle = \text{Re}[\text{Tr}(U)]/N$, which is proportional to the sum of the eigenvalues of U and provides global information about the spectral density of U . The value of the spectral density near specific energies can be obtained as shown in Fig. 2. This is like the scattering circuit except that the controlled operation U^t consists of a pair of Fourier transforms on an extra n_2 -qubit register and a controlled evolution $U^t = \exp(-iht)$ in between. The number of qubits n_2 and the timescale δ determines the resolution and range with which the spectral density is obtained. The circuit of Fig. 2 can be shown to yield $f(E) = \text{Tr}(U^t|E\rangle\langle E|\otimes\rho) = \frac{1}{N_2} \sum_{t=0}^{N_2-1} \exp(i4\pi Et/N_2) \text{Tr}(U^t\rho)$, where $N_2 = 2^{n_2}$. The function $f(E)$ is related to the spectral density at energy $e = 2E/(N_2\delta)$ by ‘smoothing’ on scales of order $2/(N_2\delta)$ and by identifying energies differing by multiples of $1/\delta$, where we have used frequency units for energy. The normalization is such that $\sum_{E=0}^{N_2-1} f(E) = 1$. This means that we can think of $f(E)$ as representing the probability that the energy content of ρ is in a region of about $\pm 1/(N_2\delta t)$ around the value $2E/(N_2\delta t)$. To be able to distinguish $f(E)$ from 0 without excessive measurement accuracy requires the energy content of ρ to be concentrated in the region considered. If such a concentration exists, it can be localized by repeating the algorithm with increasing resolution to focus in on the area of interest. Apart from being useful for localizing energy concentrations, this algorithm can be used to obtain information about the spectral density of quantum devices with fixed but unknown effects.

The method for obtaining spectral density properties can be generalized in at least two ways: first, the same ideas can be used to measure the spectral structure function, defined as the Fourier transform of $|\text{Tr}\rho U^t|^2$, characterizing the spectral correlations and the level spacing statistics of U as well as other response functions. Second, the spectrometer can also be adapted to study properties of irreversible operators. This can be done by using ancillas to simulate the environment inducing the irreversibility.

The circuit in Fig. 1 can be adapted for another important purpose: state tomography. Every time we run the algorithm for a

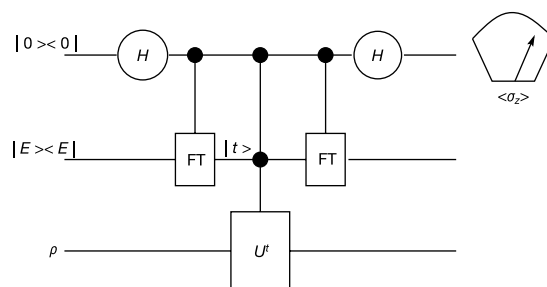


Figure 2 Circuit for evaluating the spectral density of a hamiltonian h modulated by the energy populations of the state ρ . It requires being able to conditionally realize $U^t = \exp(-iht)$. Time t is expressed in units of an appropriately chosen scale δ as indicated by coefficient of the hamiltonian in the exponent. The second ancillary register, formed by n_2 qubits, is prepared in the initial state $|E\rangle$, where E represents the energy at which we wish to evaluate the spectral density. After the first controlled Fourier transform (FT), the logical basis states of the second register represent time. The middle controlled operation maps the computational states $|1\rangle\otimes|t\rangle_2\otimes|n\rangle_3$ into $|1\rangle\otimes|t\rangle_2\otimes U^t|n\rangle_3$, with no effect if the first qubit is in the state $|0\rangle$. The second Fourier transform completes the circuit, to enable the deterministic evaluation of the spectral density. Note that the use of the Fourier transform and not its inverse is crucial for transferring the desired signal to the output. Without the ancilla qubit and if the second register is prepared in $|0\rangle$ and measured at the end, this is a version of the phase estimation algorithm⁴. For sufficiently large n_2 , the phase estimation algorithm can yield a randomly sampled eigenvalue of h (ref. 5). The circuit shown here has the advantage that only one qubit needs to be measured. The desired spectral information is given by the qubit’s polarization—a much simpler measurement that can be performed even if we only have access to ensembles of these systems without the ability to measure individual members, such as in NMR quantum computation. See also ref. 8.

known operator U , we extract information about the state ρ . Doing so for a complete basis of operators $\{A(\alpha)\}$, we get complete information and determine the full density matrix. Different tomographic schemes are characterized by the basis of operators $A(\alpha)$ that are used. Of course, completely determining the quantum state requires an exponential amount of resources. However, evaluating any coefficient of the decomposition of ρ in a given basis can be done efficiently provided that the operators $A(\alpha)$ can be implemented by efficient circuits. Here we show how to use such a 'tomographer' to directly measure the Wigner function. The Wigner function is the basic tool to represent the state of a quantum system in phase space, the natural arena of classical physics¹¹, and has been used to understand the nature of the transition from quantum to classical¹². In the case of systems with finite dimensional state spaces, the Wigner function has interesting features that have been analysed^{13,14} and that have found application in the context of quantum computation¹⁵.

The discrete Wigner function is defined by using the operators

$$A(q, p) = \tilde{U}^q R \tilde{V}^{-p} \exp(i2\pi pq/2N) \quad (2)$$

where $\tilde{U}$ is a shift operator in the computational basis ($\tilde{U}|q\rangle = |q+1\rangle$), $\tilde{V}$ is the shift in the basis related to the computational basis via the discrete Fourier transform, R is the reflection operator ($R|n\rangle = |N-n\rangle$), and N is the dimension of the Hilbert space. The discrete Wigner function $W(q, p)$ for the state ρ is evaluated at the point (q, p) in phase space by using the scattering circuit with $U = A(q, p)$ to obtain $W(q, p) = \text{Tr}[A(q, p)\rho]/(2N) = \langle \sigma_z \rangle / (2N)$. This function is defined on the grid of $2N \times 2N$ points. It shares many properties with its continuous counterpart: it is real, it can be negative, it relates traces of operator products to phase space averages $\text{Tr}(\rho_1 \rho_2) = N \sum_{q,p} W_1(q, p) W_2(q, p)$, and it provides positive probabilities when projected on any skew line in phase space.

The quantum circuit that implements $A(q, p)$ can be decomposed into a sequence of elementary steps: the controlled- $\tilde{U}$, $\tilde{V}$ and R

operations can be implemented via efficient circuits like the ones shown in ref. 16, which require a number of elementary gates that depends polynomially on $\log(N)$. We implemented the measurement of $W(q, p)$ for $N = 4$ (two qubits) and a number of different initial states. In this case, R is a controlled not (CNOT) gate (where the control is in the least significant qubit). $\tilde{U}$ is the same CNOT followed by a bit flip in the control. Analogously, $\tilde{V}$ is a sequence of controlled phase gates. The complete circuit has at most one Toffoli gate and several two qubit gates. Figure 3 shows the results of the measurement of the Wigner function for all four computational states of a two qubit system. Ideally, $W(q, p)$ for the state $|q_0\rangle$ is non-zero only on the vertical strip at $q = 2q_0$, where it is equal to $1/2N$, and on the strip at $q = 2q_0 \pm N$, where it oscillates as $(-1)^p/2N$. These oscillations correspond to interference between the state and its mirror image created by the periodic boundary conditions¹⁵ and are analogous to the sub-Planck structures recently discussed by Zurek¹⁷. In fact, whereas the total phase space is the unit square, the Wigner function oscillates on the scale of an elementary cell with area $1/N^2$. The area covered by a pure state in our system is of order $1/N$, which is the effective Planck constant. Our tomographic scheme clearly detects these structures (Fig. 3).

To measure $W(q, p)$, we used a liquid sample of trichloroethylene dissolved in chloroform. This molecule has been used in several three qubit experiments where the proton (^1H) and two strongly coupled ^{13}C nuclei (C_1 and C_2) store the three qubits (see, for example, ref. 18). We used C_1 as our probe particle and the pair $\text{H}-\text{C}_2$ to store the state whose Wigner function we measure. The coupling constants are $J_{\text{HC}_1} = 200.8 \text{ Hz}$, $J_{\text{HC}_2} = 9.1 \text{ Hz}$ and $J_{\text{C}_1\text{C}_2} = 103.1 \text{ Hz}$, and the C_1-C_2 chemical shift is $\delta_{\text{C}_1\text{C}_2} = 908.9 \text{ Hz}$. We determined the value of $W(q, p)$ for each of the independent 16 phase space points. Each of these circuits corresponds to a different sequence of radio frequency (r.f.) pulses and delays. The number of pulses in each sequence depends on q, p and varies from 5 to 17. They take at most 100 ms to execute, which in our sample is a time much

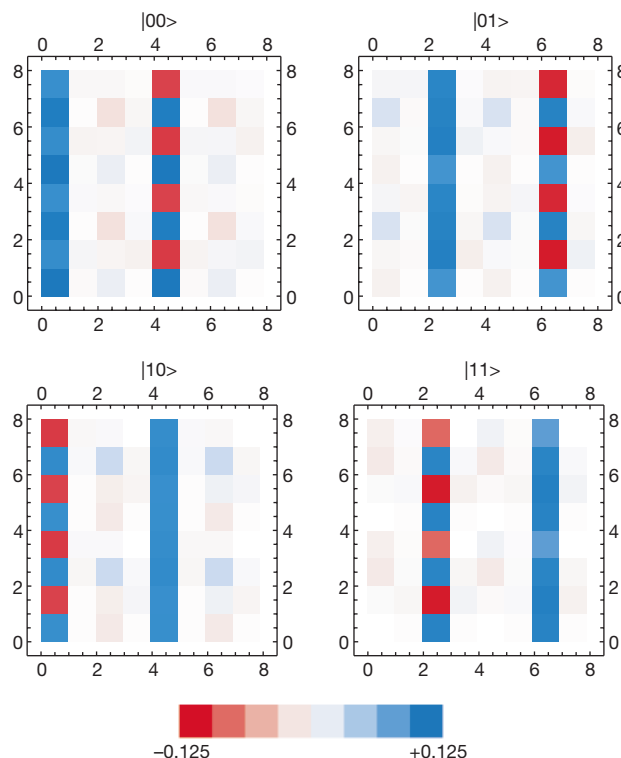


Figure 3 Measured Wigner functions for the four computational states of a two-qubit system. Horizontal and vertical axes correspond respectively to q and p coordinates. The system is based on a liquid sample of trichloroethylene in an NMR spectrometer. Ideally,

these Wigner functions should be non-zero only on two vertical strips where they take values which are $\pm 1/8$. Experimental results show small deviations from these values with a maximum error of 15%.

smaller than the relaxation and dephasing times. We used temporal averaging¹⁹ to obtain, from the part of ρ that deviates from the identity, the four pseudo-pure initial states whose Wigner functions are shown in Fig. 3. The experiments were done at room temperature on standard 500-MHz NMR spectrometers (Bruker AM-500 at the University of Buenos Aires and DRX-500 at Los Alamos National Laboratory). We used a 5-mm probe tuned to ^{13}C and ^1H frequencies of 125.8 MHz and 500.1 MHz. The most important sources of errors come from the strong coupling and the numerical uncertainty in integrating the spectra. These results illustrate the tomographic measurement of a discrete Wigner function, and agree well with theoretical expectation. The relations between this tomographic scheme and previous proposals²⁰ or actual experiments to determine the Wigner function for continuous systems^{21,22} will be discussed elsewhere¹⁵.

We have discussed a simple scattering circuit that makes it possible to see spectroscopy and tomography as dual tasks enabled by the same process. From this, we have obtained methods for characterizing spectral properties of arbitrary operators that can be applied directly when the system can be simulated on a quantum computer. Moreover, we have designed a general 'tomographer', an application of which is to directly measure the Wigner function of the state of a quantum system. $\square$

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1. Smiley, D. T., Beck, M., Reimer, M. G. & Farydani, A. Measurement of the Wigner distribution and the density matrix of a light mode using optical homodyne tomography: Application to squeezed states and the vacuum. *Phys. Rev. Lett.* **70**, 1244–1247 (1993).
2. Nielsen, M. & Chuang, I. *Quantum Information and Computation* (Cambridge Univ. Press, Cambridge, 2000).
3. Kitaev, A. Yu. Quantum measurements and the Abelian stabilizer problem. Preprint quant-ph/9511026 at (<http://xxx.lanl.gov>) (1995).
4. Lloyd, S. Universal quantum simulators. *Science* **273**, 1073–1078 (1996).
5. Shor, P. W. Polynomial-time algorithms for prime factorization and discrete logarithms on a quantum computer. *SIAM J. Comput.* **26**, 1484–1509 (1997).
6. Cleve, R., Ekert, A., Macchiavello, C. & Mosca, M. Quantum algorithms revisited. *Proc. R. Soc. Lond. A* **454**, 339–354 (1998).
7. Barenco, A. et al. Elementary gates for quantum computation. *Phys. Rev. A* **52**, 3457–3467 (1995).
8. Feynman, R. P. Simulating physics with computers. *Int. J. Theor. Phys.* **21**, 467–488 (1962).
9. Abraham, D. & Lloyd, S. Quantum algorithm providing exponential speed increase for finding eigenvalues and eigenvectors. *Phys. Rev. Lett.* **83**, 5162–5165 (1999).
10. Knill, E. & Laflamme, R. Power of one bit of quantum information. *Phys. Rev. Lett.* **81**, 5672–5675 (1999).
11. Hillery, M., O'Connell, R. F., Scully, M. O. & Wigner, E. P. Distribution functions in physics: fundamentals. *Phys. Rep.* **106**, 121–167 (1984).
12. Paz, J. P. & Zurek, W. H. in *Coherent Matter Waves, Les Houches Session LXXII* (eds Kaiser, R., Westbrook, C. & David, F.) 533–614 (EDP Sciences, Springer, Berlin, 2001).
13. Wootters, W. K. A Wigner function formulation for finite state quantum mechanics. *Ann. Phys. NY* **176**, 1–21 (1987).
14. Leonhardt, U. Discrete Wigner function and quantum-state tomography. *Phys. Rev. A* **53**, 2998–3013 (1996).
15. Miquel, C., Paz, J. P. & Saraceno, M. Quantum computers in phase space. *Phys. Rev. A* (in the press); preprint quant-ph/0204149 at (<http://xxx.lanl.gov>) (2002).
16. Miquel, C., Paz, J. P. & Perazzo, R. Factoring in a dissipative quantum computer. *Phys. Rev. A* **54**, 2605–2613 (1996).
17. Zurek, W. H. Sub-Planck structure in phase space and its relevance for quantum decoherence. *Nature* **412**, 712–717 (2001).
18. Cory, D. G. et al. Experimental quantum error correction. *Phys. Rev. Lett.* **81**, 2152–2155 (1998).
19. Knill, E., Chuang, I. & Laflamme, R. Effective pure states for bulk quantum computation. *Phys. Rev. A* **57**, 3348–3363 (1998).
20. Lutterbach, L. G. & Davidovich, L. Method for direct measurement of the Wigner function in cavity QED and ion traps. *Phys. Rev. Lett.* **78**, 2547–2550 (1997).
21. Lvovsky, L. et al. Quantum state reconstruction of the single-photon Fock state. *Phys. Rev. Lett.* **87**, 402–405 (2001).
22. Nogue, G. et al. Measurement of a negative value for the Wigner function of radiation. *Phys. Rev. A* **62**, 4101–4104 (2000).

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Mechanism of hydrogen-induced crystallization of amorphous silicon

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Hydrogenated amorphous and nanocrystalline silicon films manufactured by plasma deposition techniques are used widely in electronic and optoelectronic devices^{1,2}. The crystalline fraction and grain size of these films determines electronic and optical properties; the nanocrystal nucleation mechanism, which dictates the final film structure, is governed by the interactions between the hydrogen atoms of the plasma and the solid silicon matrix. Fundamental understanding of these interactions is important for optimizing the film structure and properties. Here we report the mechanism of hydrogen-induced crystallization of hydrogenated amorphous silicon films during post-deposition treatment with an H_2 (or D_2) plasma. Using molecular-dynamics simulations^{3,4} and infrared spectroscopy⁵, we show that crystallization is mediated by the insertion of H atoms into strained Si–Si bonds as the atoms diffuse through the film. This chemically driven mechanism may be operative in other covalently bonded materials, where the presence of hydrogen leads to disorder-to-order transitions.

A fundamental understanding of chemically induced disorder-to-order structural transitions is relevant to the synthesis of various technologically important materials, including hydrogenated amorphous (a-Si:H) and nanocrystalline (nc-Si:H) silicon films that are used in solar cells, displays and imaging devices^{1,2}. Thin a-Si:H films crystallize at temperatures much lower than those required for thermal crystallization upon exposure to H created through plasma dissociation of H_2 (refs 6–11). Plasma deposition from SiH_4 gas heavily diluted in H_2 also results in nc-Si:H films where nanocrystals are either abutted against each other¹² or embedded in an amorphous matrix¹³. Surface diffusion^{14,15} and selective etching^{7,10,16} models have been proposed to explain nc-Si:H deposition. In the surface diffusion model, high H flux impinging on the surface is thought to enhance the mobility of the deposition precursors. In the selective etching model, amorphous and crystalline phases are assumed to be deposited simultaneously, but H selectively etches the amorphous material leaving behind a nc-Si:H film. However, these mechanisms do not explain the crystallization of an a-Si:H film by post-deposition H exposure. Chemical annealing has been proposed as a mechanism where H aids crystallization by annihilating the strained Si–Si bonds in the a-Si:H film^{9,17–19}. Nevertheless, the atomic-scale mechanisms that result in H-induced crystallization remain unclear.

To determine the mechanism of H-induced crystallization of amorphous silicon, we investigated the structural evolution of a-Si:H films upon exposure to H (and/or D) atoms through a combination of molecular-dynamics (MD) simulations and *in situ* attenuated total-reflection Fourier-transform infrared spectroscopy (ATR–FTIR). We discovered that H-induced crystallization is mediated by insertion of H atoms into strained Si–Si bonds through the formation of intermediate bond-centred Si–H–Si configurations as the H atoms diffuse through the a-Si:H film. This configuration is observed during crystallization both in the MD simulations and in the infrared spectra of the D-exposed a-Si:H films. As the H atoms move through the a-Si:H matrix, they either break or perturb strained Si–Si bonds. Subsequent structural relaxation of these Si–Si bonds results in the transformation of

the film's structure from amorphous to nanocrystalline.

Exposure of the a-Si:H film to H from an H₂ plasma was simulated by MD through repeated impingement of H atoms at normal incidence onto the a-Si:H film surface. The chemical reactions and transport phenomena observed during the MD simulations of H impingement on a-Si:H films include: (1) H adsorption onto the surface; (2) surface H abstraction by the impinging H atoms; (3) insertion of H into strained Si-Si bonds; (4) H diffusion into the bulk substrate/film; (5) sputtering of surface H atoms; and (6) desorption into the gas phase of H from surface silicon hydrides with overcoordinated Si atoms. Events (1)–(4) were the most frequently observed in the simulations; their occurrence was verified through *in situ* ATR-FTR spectroscopy during D(H) exposure of a-Si:H(D) films. Most significant among these reactions is the insertion of H into Si-Si bonds, which results in structural changes in the film and eventually in the appearance of nanocrystalline domains within the a-Si:H films. These structural changes are most evident in the evolution of the Si-Si pair correlation function, $g(r)$, as the H exposure proceeds (Fig. 1). The $g(r)$ for a crystalline Si (c-Si) substrate exhibits peaks corresponding to the sequence of coordination shells that are characteristic of the crystalline material's short- and long-range order. In contrast, the corresponding Si-Si $g(r)$ for the a-Si:H film before exposure to H atoms exhibits only the first two peaks (blue curve 1 in Fig. 1). With increasing H exposure, this Si-Si $g(r)$ of a-Si:H evolves toward that of c-Si, exhibiting peaks that correspond to the third, fourth, fifth, sixth and higher crystalline coordination shells. The appearance of these structural features indicates unambiguously that the a-Si:H film becomes progressively more crystalline when exposed to H atoms. Similar results were obtained from MD simulations with H atoms impinging over a range of kinetic energies (0.04–5.0 eV), indicating that the mechanism of the disorder-to-order transition is independent of the kinetic energy of the impinging H atoms over the range studied.

A detailed structural analysis of the H-exposed a-Si:H film revealed ordered regions (nc-Si:H) that are embedded within the a-Si:H matrix (Fig. 2a, b, d and e). A comparison of the pre- (Fig. 2a and d) and post- (Fig. 2b and e) H-exposed structures in these regions with those for c-Si (Fig. 2c and f) clearly shows the transformation from a-Si:H to nc-Si:H, with some unhealed structural defects still remaining at the interface between the amorphous and crystalline regions. The structural evolution in the transformed regions as a function of the H-exposure time reveals that the amorphous-to-crystalline transition is mediated by relaxation of

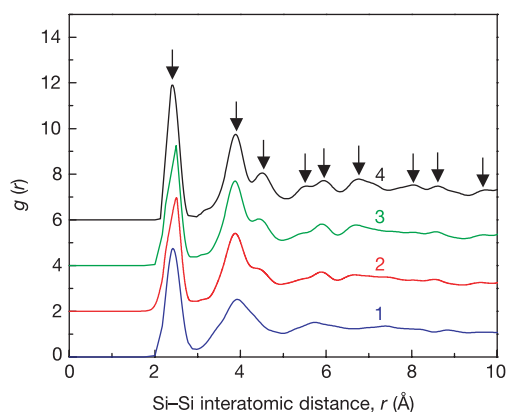


Figure 1 Simulated evolution of the Si-Si pair correlation function, $g(r)$, of the a-Si:H film with increasing H exposure. The numbering of the correlation functions increases with increasing H exposure. Curve 1, $t = 0.0$ ns; curve 2, $t = 0.36$ ns; (3) $t = 0.58$ ns, and (4) $t = 0.80$ ns, where t is time in the molecular-dynamics simulation of H exposure. The Si-Si $g(r)$ of a-Si:H evolves towards that of c-Si and begins to exhibit peaks that correspond to the higher crystalline coordination shells. The arrows indicate the peak positions in the $g(r)$ of c-Si.

strained Si-Si bonds as a result of H insertion into these bonds. During diffusion through the film, H inserts into strained Si-Si bonds, forming intermediate bond-centred Si-H-Si configurations^{20,21}. After the H moves away from the bond-centred location, the strained Si-Si bonds either break or relax, then undergo local structural rearrangements that result in bond lengths and angles closer to those of c-Si.

Structural disorder arising from coordination defects (Fig. 2a, d, and g) is commonly found in a-Si:H. Energetically more stable and ordered structures resembling c-Si are formed when such defects are annealed as a result of H interaction with strained bonds, through a combination of insertion by bond breaking, bond reformation and network rearrangement processes (Fig. 2). One such representative local structural rearrangement is shown in Fig. 2g and h, where many H-insertion reactions in the vicinity of a representative Si cluster in the simulation cell cause Si-Si bond relaxation, leading eventually to atomic displacements toward the equilibrium c-Si lattice positions. Following H-insertion events, the Si-Si bonds relax, which leads to structural ordering of the Si cluster through the concerted atomic displacements of the Si atoms numbered from 1 to 8. As a result of the atomic displacements, a coordination defect present in the form of a seven-membered ring (atoms 2 to 8) is eliminated, resulting in a structure which resembles that of c-Si (Fig. 2i). The superposition of the black dots that indicate the c-Si lattice positions onto the Si cluster structure, obtained after many H

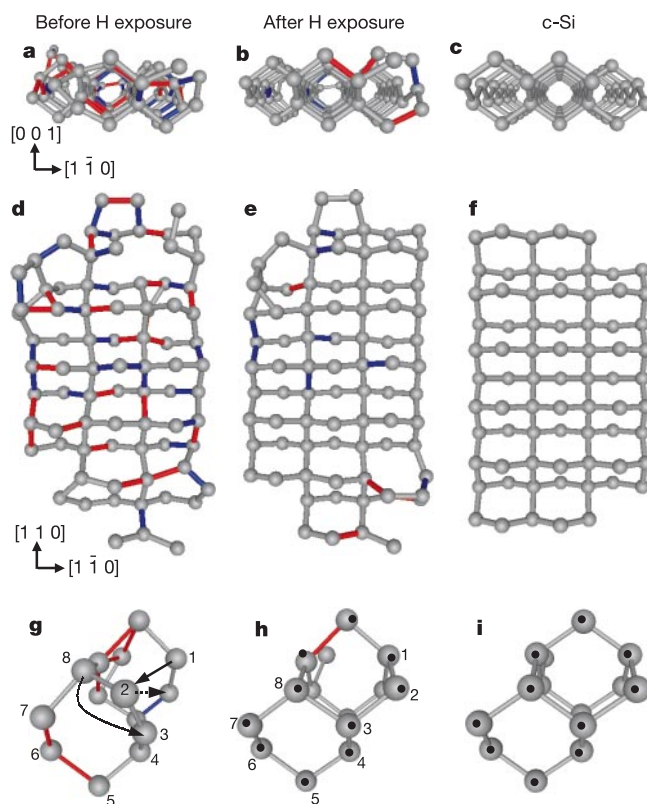


Figure 2 Simulated structural characteristics of Si film before and after H exposure. The region of the simulation cell that transforms from amorphous to nanocrystalline Si as viewed from the [110] (a and b) and [001] (d and e) directions, before (a and d) and after (b and e) H exposure. Representative local structural rearrangements in the Si film induced by H exposure and the resulting configuration are shown in g and h, respectively. The dashed arrow in g indicates the direction in which atom numbered 2 moves, whereas the solid arrows indicate the atoms that bind to each other upon H-induced relaxation. The Si-Si bonds under tensile and compressive strain (bond lengths greater than 2.6 Å and less than 2.3 Å, respectively) are indicated by red and blue, respectively. The equilibrium bond length in c-Si is 2.35 Å. The corresponding c-Si clusters are shown in c, f and i for comparison. For clarity, the H atoms are not shown.

interaction events, shows that the rearranged atomic positions in the Si cluster correspond well with the equilibrium c-Si lattice sites.

We established the presence of bond-centred H formed through the insertion of H into strained Si–Si bonds both through MD simulations and through *in situ* ATR–FTIR spectroscopy. The distribution of Si–H bond lengths obtained from MD simulations during the H exposure shows a population centred at 1.65 Å (Fig. 3a) that corresponds to the bond-centred Si–H–Si configuration; this is in addition to the normal population of Si–H bonds in a-Si:H centred at 1.51 Å. The bond lengths and bond angles in the bond-centred configurations compare well with targeted density functional theory (DFT) calculations of Tuttle and Adams within the local density approximation²¹. Evidence for the existence of bond-centred H(D) was found in our experiments where a-Si:H films

grown by plasma-assisted chemical vapour deposition were exposed to D (or H) atoms from a D₂ (or H₂) plasma. Bond-centred H and D have not been previously observed in a-Si:H. The Si–H absorption in the Si–H–Si configuration is expected at 1,945 cm^{−1} according to DFT calculations within the local density approximation²⁰. A distinct absorption band for Si–H–Si is not observed in the infrared spectra of a-Si:H owing to the broad bulk Si–H stretching absorption in the Si films centred at 2,000 cm^{−1}. However, when an a-Si:H film is exposed to D atoms from a D₂ plasma, Si–D–Si configurations are formed at a rate comparable to the abstraction and replacement of H in isolated bulk Si–H with D. The infrared spectrum of the as-deposited a-Si:H film was used as a reference so that the differential increase in various Si–D stretching absorptions could be observed directly. Thus, the use of D instead of H enables clear detection of the Si–D–Si configuration as a small but clear shoulder next to the bulk Si–D absorption as shown in Fig. 3b.

The Si–D absorption band was deconvoluted using seven gaussian absorption peaks corresponding to the stretching modes of Si–D–Si and of surface and bulk silicon deuterides on the basis of well known peak assignments for Si–D(H) absorptions in c-Si, a-Si:H and a-Si:D^{5,22}. The Si–D absorption in the Si–D–Si configuration is expected to shift by a factor of 0.73 to 1,420 cm^{−1}; this shift is based on the experimentally measured ratio of the isolated Si–D and Si–H absorption frequencies in a-Si:H and a-Si:D. In the infrared absorption spectrum of a-Si:H films exposed to D atoms from a D₂ discharge, we find a clear broad shoulder centred at 1,423 ± 4 cm^{−1} corresponding to the expected location for the Si–D absorptions in Si–D–Si. Attempts to fit the spectrum without an absorption peak at 1,423 cm^{−1} result in a large residue in this region as shown in the inset of Fig. 3b. Furthermore, the residual in this region is not sensitive to the changes in the positions of the peaks 3–7.

The H-insertion reactions into the bond-centred location facilitate rearrangement of the amorphous Si network to crystallize even at very low temperatures where thermal annealing is expected to be too slow to lead to nucleation and growth of nanocrystalline silicon. This chemically induced ordering mechanism is in contrast to thermal annealing, which leads to crystallization as a result of thermal fluctuations at high annealing temperatures. In the H-induced disorder-to-order transformation, chemical reactions of H atoms with Si–Si bonds provide new pathways through which Si atoms can rearrange. Specifically, bond breaking and reforming reactions are facilitated by H insertion, which results in elimination of strained Si–Si bonds and concerted atomic rearrangements, such as those shown in Fig. 2. These atomic-scale mechanisms play an important role in the nucleation and growth of the nanocrystalline Si structure. Finally, the mechanism elucidated through this study of a-Si:H to nc-Si:H transformation may have implications for other systems where the presence of H leads to disorder-to-order transitions²³. □

Methods

The H(D) exposure experiments were conducted in a high-vacuum, inductively coupled plasma reactor using *in situ* ATR–FTIR⁵. The details of the experimental set-up have been described in ref. 5. The a-Si:H films were deposited in the same chamber from an SiH₄ (1% in Ar) discharge at a substrate temperature of 573 K. The as-deposited films were exposed to D atoms produced by electron-impact dissociation of D₂ in the plasma at 573 K. The resulting changes in Si–H and Si–D bonding were monitored through the evolution of the infrared absorbance spectra collected in the ATR geometry. The use of D₂ instead of H₂, in conjunction with spectra collection in the differential mode, allows for the monitoring of H abstraction and subsequent passivation of the dangling bonds with D, as well as detection of D insertion without interference from Si–H absorptions already existing in the film. The stretching modes for silicon hydrides and silicon deuterides appear at ~2,100 cm^{−1} and ~1,500 cm^{−1}, respectively, and can be resolved easily.

The interatomic interactions in the MD simulations were described by an extension of Tersoff's empirical potential for Si, to include Si–H, H–H, and the corresponding three-body interactions^{24,25}. The interatomic potential has been extensively tested and compared with experimental data and *ab initio* calculations within DFT for the structure and properties of bulk a-Si:H, the structure and energetics of the pristine and H-terminated Si(001)–(2 × 1) surfaces and the gas-phase and gas-surface interaction chemistry of SiH_x species^{3,26–30}. The a-Si:H films were deposited by MD on an initial H-terminated Si(001)–

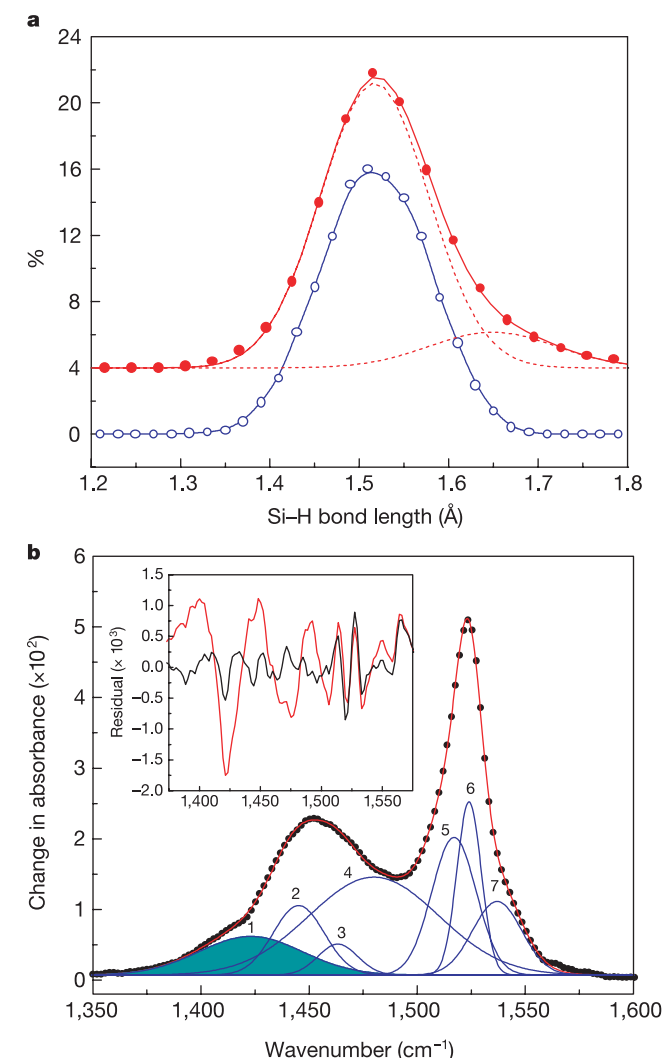


Figure 3 Simulation and experimental evidence for the existence of the Si–H–Si (Si–D–Si) configuration. **a**, Comparison of Si–H bond-length distributions before (blue line) and after (red) H exposure as obtained from MD simulations. The populations of Si–H bond lengths centred at 1.51 Å and 1.65 Å are shown as dashed red lines. The distribution after H exposure is shifted in the ordinate by 4% for clarity. **b**, Infrared spectrum (filled circles) of an a-Si:H film after exposure to D atoms at 573 K. The red line is the sum of all the blue absorption peaks (1–7) used to fit the spectrum. The Si–D absorption in the Si–D–Si configuration appears as a shoulder (shaded peak 1) centred at 1,423 cm^{−1}. The inset shows the residual (the difference between the experimental and fitted spectra) with (black) and without (red) the peak at 1,423 cm^{−1}. The peaks numbered 2–4 at 1,445 cm^{−1}, 1,463 cm^{−1} and 1,480 cm^{−1} correspond to bulk Si–D vibrational modes, whereas peaks 5–7 at 1,516 cm^{−1}, 1,525 cm^{−1}, and 1,537 cm^{−1} correspond to surface SiD, SiD₂ and SiD₃, respectively.

(2 × 1) substrate surface through repeated impingement of SiH₃ radicals as the sole precursor at a substrate temperature of 773 K (refs 3, 4). Exposure of these a-Si:H films to H from an H₂ plasma was simulated by MD through repeated impingement of H atoms over a range of kinetic energies (0.04–5.0 eV) at normal incidence onto the a-Si:H film surface. The results presented in this article correspond to H atoms with a kinetic energy of 5 eV. The time interval between all successive impingement events was fixed at 4 ps, a period sufficient to attain surface relaxation^{3,4,25}.

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- Shah, A., Torres, P., Tscharnner, R., Wyrsh, N. & Keppner, H. Photovoltaic technology: the case for thin-film solar cells. *Science* **285**, 692–698 (1999).
- Street, R. A. Large area electronics, applications and requirements. *Phys. Status Solidi A* **166**, 695–705 (1998).
- Maroudas, D. Modeling of radical-surface interactions in the plasma-enhanced chemical vapor deposition of silicon thin films. *Adv. Chem. Eng.* **28**, 251–296 (2001).
- Ramalingam, S., Sriraman, S., Aydil, E. S. & Maroudas, D. Evolution of structure, morphology, and reactivity of hydrogenated amorphous silicon film surfaces grown by molecular-dynamics simulation. *Appl. Phys. Lett.* **78**, 2685–2687 (2001).
- Marra, D. C., Edelberg, E. A., Naone, R. L. & Aydil, E. S. Silicon hydride composition of plasma-deposited hydrogenated amorphous and nanocrystalline silicon films and surfaces. *J. Vac. Sci. Technol. A* **16**, 3199–3210 (1998).
- Abelson, J. R. Plasma deposition of hydrogenated amorphous silicon: studies of the growth surface. *Appl. Phys. A* **56**, 493–512 (1993).
- Layadi, N., Roca i Cabarrocas, P., Devillon, B. & Solomon, I. Real-time spectroscopic ellipsometry study of the growth of amorphous and microcrystalline silicon thin films prepared by alternating silicon deposition and hydrogen plasma treatment. *Phys. Rev. B* **52**, 5136–5143 (1995).
- Saitoh, K. *et al.* Role of the hydrogen plasma treatment in layer-by-layer deposition of microcrystalline silicon. *Appl. Phys. Lett.* **71**, 3403–3405 (1997).
- Kaiser, I., Nickel, N. H., Fuhs, W. & Pilz, W. Hydrogen-mediated structural changes of amorphous and microcrystalline silicon. *Phys. Rev. B* **58**, R1718–R1721 (1998).
- Fontcuberta i Morral, A., Bertomeu, J. & Roca i Cabarrocas, P. The role of hydrogen in the formation of microcrystalline silicon. *Mater. Sci. Eng. B* **69**, 559–563 (2000).
- Summonte, C. *et al.* Plasma-enhanced chemical vapour deposition of microcrystalline silicon: on the dynamics of the amorphous-microcrystalline interface by optical methods. *Phil. Mag. B* **80**, 459–473 (2000).
- Edelberg, E., Bergh, S., Naone, R., Hall, M. & Aydil, E. S. Luminescence from plasma deposited silicon films. *J. Appl. Phys.* **81**, 2410–2417 (1997).
- Roca i Cabarrocas, P. Plasma enhanced chemical vapor deposition of amorphous, polymorphous and microcrystalline silicon films. *J. Non-Cryst. Solids* **266**, 31–37 (2000).
- Nomoto, K., Urano, Y., Guizot, J. L., Ganguly, G. & Matsuda, A. Role of hydrogen atoms in the formation process of hydrogenated microcrystalline silicon. *Jpn J. Appl. Phys.* **29**, L1372–L1375 (1990).
- Katayar, M. & Abelson, J. R. Investigation of hydrogen induced phase transition from a-Si:H to μ -c-Si:H using real time infrared spectroscopy. *Mater. Sci. Eng. A* **304**, 349–352 (2001).
- Tsai, C. C., Anderson, G. B., Thompson, R. & Wacker, B. Control of silicon network structure in plasma deposition. *J. Non-Cryst. Solids* **114**, 151–153 (1989).
- Boland, J. J. & Parsons, G. N. Bond selectivity in silicon film growth. *Science* **256**, 1304–1306 (1992).
- Nickel, N. H. & Jackson, W. B. Hydrogen-mediated creation and annihilation of strain in amorphous silicon. *Phys. Rev. B* **51**, 4872–4881 (1995).
- Shirai, H., Hanna, J. & Shimizu, I. Role of atomic hydrogen during growth of hydrogenated amorphous silicon in the 'chemical annealing'. *Jpn J. Appl. Phys.* **30**, L679–L682 (1991).
- Van de Walle, C. G., Denteneer, P. J. H., Bar-Yam, Y. & Pantelides, S. T. Theory of hydrogen diffusion and reactions in crystalline silicon. *Phys. Rev. B* **39**, 10791–10808 (1989).
- Tuttle, B. & Adams, J. B. Energetics of hydrogen in amorphous silicon: An ab initio study. *Phys. Rev. B* **57**, 12859–12868 (1998).
- Chabal, Y. J. Surface infrared spectroscopy. *Surf. Sci. Rep.* **8**, 211–357 (1988).
- Jun, X. *et al.* Device-grade a-SiGe:H alloys prepared by nanometer deposition/H₂ plasma annealing method. *J. Non-Cryst. Solids* **200**, 582–586 (1996).
- Terzoff, J. New empirical approach for the structure and energy of covalent systems. *Phys. Rev. B* **37**, 6991–7000 (1988).
- Ohira, T., Ukai, O., Adachi, T., Takeuchi, Y. & Murata, M. Molecular-dynamics simulations of SiH₃ radical deposition on hydrogen-terminated silicon (100) surfaces. *Phys. Rev. B* **52**, 8283–8287 (1995).
- Ramalingam, S., Maroudas, D. & Aydil, E. S. Interactions of SiH radicals with silicon surfaces: An atomic-scale simulation study. *J. Appl. Phys.* **84**, 3895–3911 (1998).
- Ramalingam, S., Maroudas, D. & Aydil, E. S. Atomistic simulation study of the interactions of SiH₃ radicals with silicon surfaces. *J. Appl. Phys.* **86**, 2872–2888 (1999).
- Ramalingam, S., Maroudas, D., Aydil, E. S. & Walch, S. P. Abstraction of hydrogen by SiH₃ from hydrogen-terminated Si(001)-(2 × 1) surfaces. *Surf. Sci.* **418**, L8–L13 (1998).
- Walch, S. P., Ramalingam, S., Aydil, E. S. & Maroudas, D. Mechanism and energetics of dissociative adsorption of SiH₃ on the hydrogen-terminated Si(001)-(2 × 1) surface. *Chem. Phys. Lett.* **329**, 304–310 (2000).
- Walch, S. P., Ramalingam, S., Sriraman, S., Aydil, E. S. & Maroudas, D. Mechanism and energetics of SiH₃ adsorption on the pristine Si(001)-(2 × 1) surface. *Chem. Phys. Lett.* **344**, 249–255 (2001).

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Muted climate variations in continental Siberia during the mid-Pleistocene epoch

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The large difference in carbon and oxygen isotope data from the marine record between marine oxygen isotope stage 12 (MIS 12) and MIS 11, spanning the interval between about 480 and 380 kyr ago, has been interpreted as a transition between an extremely cold glacial period and an unusually warm interglacial period, with consequences for global ice volume, sea level and the global carbon cycle^{1–4}. The extent of the change is intriguing, because orbital forcing is predicted to have been relatively weak at that time⁵. Here we analyse a continuous sediment record from Lake Baikal, Siberia, which reveals a virtually continuous interglacial diatom assemblage, a stable littoral benthic diatom assemblage and lithogenic sediments with 'interglacial' characteristics for the period from MIS 15a to MIS 11 (from about 580 to 380 kyr ago). From these data, we infer significantly weaker climate contrasts between MIS 12 and 11 than during more recent glacial–interglacial transitions in the late Pleistocene epoch (about 130 to 10 kyr ago). For the period from MIS 15a to MIS 11, we also infer an apparent lack of extensive mountain glaciation.

Here we examine the response of the world's largest continental interior to climate conditions during MIS 12 and MIS 11; this response can usefully be compared with marine records. Because the contrast between cooling over land masses and warm oceans is essential to generate glaciations⁶, continental areas have potential for recording the history and intensity of past glaciations. Likewise, multiple proxies of terrestrial and aquatic biological productivity on continents record the amplitudes and duration of past interglacials. The sharp seasonal insolation gradients contributing to global glacial inception and terminations are induced by the precessional orbital cycle through terrestrial processes⁷. These processes have short response times, and operate primarily in the areas of the Northern Hemisphere that are most sensitive to seasonal insolation changes⁷. Interior Asia, globally the region most sensitive to precessional insolation forcing⁸, has an increased responsiveness that makes it useful for recording the amplitudes of past climatic variations. The interior of Asia has never been fully glaciated, and has preserved continuous Pleistocene sedimentary archives.

We use the new refined age model⁹ for the continuous 500-year-resolution sedimentary record from Lake Baikal¹⁰ as a gauge of the amplitude, timing and duration of Northern Hemisphere glacial/interglacial climatic and environmental changes. Based on a robust palaeomagnetic event/reversal age scale^{10,11}, the Baikal record shows strong variance in Milankovitch frequency bands^{10,12}, and is highly correlative with long-term global palaeoclimate records^{9–13}. In addition, despite its mid-continent location, Baikal is also responsive to millennial sub-Milankovitch climatic events recorded in Europe and the North Atlantic Ocean^{14,15}.

The mid-Pleistocene sedimentary record at the pelagic BDP-96

drill site reflects primarily climate-driven changes in terrigenous and biogenic sediment fluxes¹⁶. Tectonic processes are unlikely to have significantly biased the productivity records in Baikal during the Brunhes chron. The most active tectonic phase, which largely shaped the present-day regional geomorphology, ended about 2.5 Myr ago—as indicated by sedimentary sequence morphology and structure, and as reflected in Baikal sedimentary records^{17–19}. There are no indications that local tectonic block movements around 1–1.3 Myr ago¹⁸ and the shift of regional volcanism to the periphery of the Baikal rift zone about 600–700 kyr ago²⁰ signifi-

cantly affected Baikal sedimentary records. (There is a lack of trends in the biogenic silica record¹⁰ and in magnetic susceptibility¹¹ during the interval 1.3–1.5 Myr ago, both before and after the MIS 12–11 interval.) Although we are cautious not to interpret the biogenic silica palaeoproductivity record as a direct climate index⁹, we nevertheless find that Baikal proxy records adequately reflect the magnitude of climate-induced changes in the watershed and the lake itself.

Before discussing the details of the mid-Pleistocene signals, we show that Baikal proxies effectively work as a Northern Hemisphere

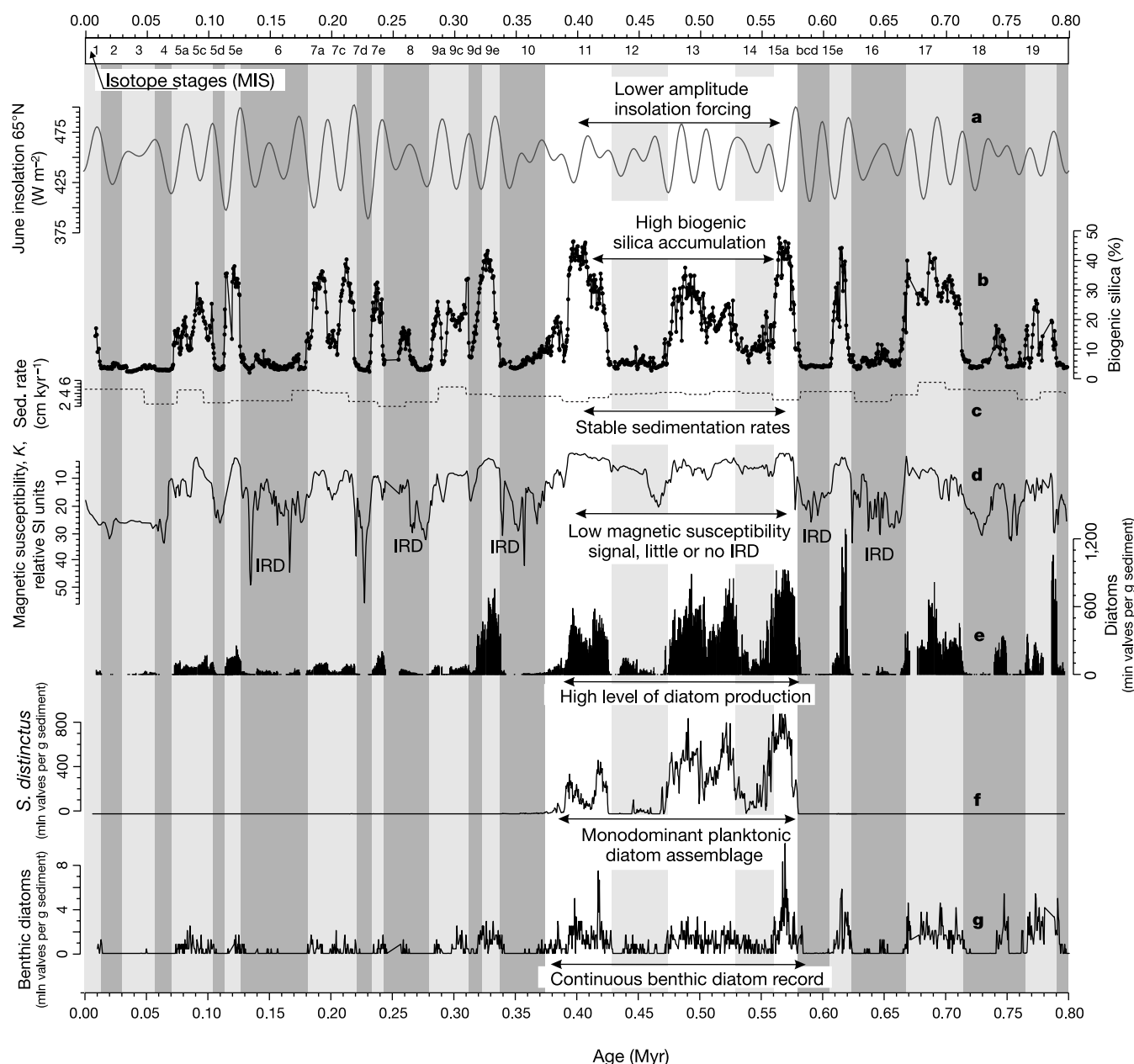


Figure 1 Lake Baikal palaeoclimate proxy records of the Brunhes chron. All records are from drill core BDP-96-2 (53° 41' 48" N, 108° 21' 06" E, water depth 321 m), plotted to an age scale in millions of years before present (Myr). The June insolation curve at 65° N (a), Laskar 1.1 solution²⁹, was used as a tuning target for the age scale of the biogenic silica record⁹ (b). Darker shading indicates glacial periods in the Lake Baikal region, including even-numbered marine oxygen isotope stages (MIS) and substages 5d, 7d, 9d and 15bcd. The interval from MIS 15a to MIS 11 with light shading shows high interglacial levels of biogenic silica accumulation, indicative of warm climate optima. Apparent

sedimentation rates are relatively stable in this interval (c). The magnetic susceptibility record (d) reveals particularly low 'interglacial' values during MIS 15a–11, and reflects a lack of glacial IRD signals during MIS 14 and 12. Supportive of the lack of extreme climatic contrasts during MIS 15a–11 in comparison with other Pleistocene glacials are high levels of diatom production in Lake Baikal (e), the monospecific interglacial planktonic diatom assemblage (f), and the mainly uninterrupted development of the littoral benthic diatom assemblage (g).

continental 'palaeoclimate gauge'. For example, following the basin-wide maximum of diatom production during the last interglacial (MIS 5e), accumulation of biogenic silica in Baikal abruptly declined at the time of the Northern Hemisphere glacial inception¹³ (Fig. 1b). In sharp contrast to interglacial sediment type, glacial sediments in Baikal consist of fine, diatom-barren, glacial clay with iceberg-rafted detritus (IRD) produced by mountain glaciers descending into the lake¹³. The precession-controlled structure of MIS 5, 7 and 9 intervals (Fig. 1) indicates that periods of high diatom productivity during Siberian interglacials alternated with rapid regional glaciation events coeval with—or perhaps even leading—the initiation of global glacial periods^{9,13}. Glacial IRD produces distinct spikes in the Baikal magnetic susceptibility records: for instance, during MIS 6, 7d, 8 and 10 (Fig. 1d). Thus, Baikal proxy records are of adequate sensitivity and resolution to derive the Asian perspective on the glacial/interglacial climate extremes in the Northern Hemisphere during the mid-Pleistocene. As we discuss below, this perspective differs significantly from some of the implications of marine records.

The highest peaks of biogenic silica and diatom abundance in Baikal during the Brunhes chron, reflecting the highest lacustrine productivity levels, correspond to MIS 15 and 11 (Fig. 1b and e). In addition, the interval of ~580–380 kyr ago, encompassing interglacials MIS 15a, 13 and 11, is characterized by a distinct structure in the profiles of biogenic silica and diatom abundance. Unlike the Baikal interglacial stages of the last three climatic cycles, which are punctuated by rapid coolings and regional glaciations in response to the precessional forcing (for example, MIS 5b, 5d, 7b, 7d, 9b, 9d)^{9,13}, the MIS 11 and 13 intervals reveal sustained levels of biogenic silica accumulation (Fig. 1b). As compared with the last three climatic cycles, this response is apparently indicative of lesser amplitudes of climatic variations in southeast Siberia.

The distinct MIS 15a–11 interval is bounded above and below by diatom-barren and IRD-rich glacial intervals of MIS 10 and 15bcd (Fig. 1d and e). But within the MIS 15a–11 interval, the glacials do not appear to be so severe. Usually, Pleistocene glaciations, including the short MIS 5d, 7d and 15bcd intervals, led to ecological catastrophes resulting in dramatic drops of Baikal diatom production. Following such glacial effects, the lacustrine system was able to recover not earlier than the next interglacial (Fig. 1b and e). Yet the diatom-barren portion of MIS 12 is rather short: diatom accumulation in Baikal, although significantly reduced, continued throughout the most of MIS 12 (Fig. 1e). The MIS 14 glacial is even more unusual: biogenic silica and diatom accumulation were continuous at relatively high interstadial levels throughout this entire interval (Fig. 1b, e). From the observed differences between the Baikal proxy responses during MIS 12 and 14 and during other Pleistocene glacials, we conclude that MIS 12 and 14 were not among the strongest regional glacial episodes during the past 800,000 years.

The examination of diatom species composition further reinforces this conclusion. Climate change is important in controlling diatom flora renewal and extinctions, as seen from changes in the Baikal diatom palaeo-assemblages during the Brunhes chron²¹ and from diatom extinctions at times of most severe regional climatic deteriorations, such as climatic transitions MIS 5/4, 9e/9d, 11/10, 15e/15d, 17/16 and 19/18²¹. In each of these cases, the subsequent return to interglacial conditions resulted in the development of a renewed assemblage of Baikal pelagic flora. From this perspective, the Baikal MIS 15a–11 interval is exceptional, because the species composition of diatom flora encompassing three interglacial and two glacial intervals remained virtually unchanged for as long as 200,000 years²¹. Moreover, the diatom flora from MIS 15a to the middle of MIS 11 was almost monospecific, with the species *Stephanodiscus distinctus* dominant throughout the entire interval²¹ (Fig. 1f). The unchanging composition of this monospecific diatom assemblage suggests that

environmental impacts during the MIS 12 and 14 glacials were not as extreme as during other Pleistocene glacials, representing major climatic-biostratigraphic boundaries.

Further evidence supporting the lack of an extreme nature of MIS 12 and 14 comes from the benthic diatom record (Fig. 1g). Although not abundant at the pelagic BDP-96 drill site, benthic diatoms reflect productivity in the littoral zone. In addition to their effect on the pelagic ecosystem in Baikal, most cold Pleistocene intervals also had a great effect on the littoral zone, resulting in the absence of benthic diatoms during cold intervals (Fig. 1g). In contrast to 'typical' Baikal glacials, MIS 14 and MIS 12 have a record of mainly uninterrupted benthic diatom accumulation. In addition, the abundance of benthic diatoms during MIS 15e–11, similar to that of planktonic diatoms (Fig. 1e), is the highest for the entire Brunhes chron (Fig. 1g). Prolonged conditions favourable to benthic diatom development in the Baikal littoral zone during the mid-Pleistocene suggest lesser relative amplitudes in climate-driven environmental changes, such as temperature, nutrient supply, and light transmission controlled by suspended solids. Therefore, the continuity of the benthic diatom record further corroborates the conclusion that although the Baikal region did experience significant climatic changes during the MIS 15a–11 interval, their amplitudes were not as dramatic as during several earlier and later glacial/interglacial cycles.

The rock-magnetic signature of Baikal's mid-Pleistocene interval is also exceptional, as seen from low magnetic susceptibility values (Fig. 1d). For the late MIS 12 and the entire MIS 14 interval, magnetic susceptibility is 'fully interglacial' by Baikal standards. For example, the magnitude of this signal during early MIS 12 is much weaker than during any other glacial stage (Fig. 1d). Because glacial sediments form during periods of suppressed soil development and weathering, they are expected to reflect the primary magnetic mineralogy of the exposed parent material in the catchment basin²². The magnetic susceptibility signal in a series of sedimentary records of the last climatic cycle from the Academician ridge of Lake Baikal¹⁶ including BDP-96-2 (Fig. 1d) is much stronger than during glacial MIS 12 and 14. The lack of 'fresh' parent material during MIS 12 and 14, as indicated by the character of the rock-magnetic signal, further implies that the inorganic component of Baikal sediments during MIS 12 and 14 was not typical glacial parent material. Markedly low susceptibility signals may indicate that physical erosion rates in the Baikal catchment basin, typically high during glacials, slowed down during this portion of the mid-Pleistocene. An additional distinguishing characteristic of MIS 12 and 14 is the absence of high-susceptibility IRD spikes, indicating that the development of mountain glaciers discharging into the lake was rather limited during these glacials. Thus, the magnetic susceptibility record corroborates several other lines of evidence that glacial/interglacial climatic contrasts in the Baikal region during this part of the mid-Pleistocene were not as extreme as during earlier and later parts of the Brunhes chron.

The Lake Baikal record, representing the largest continental interior region on Earth, offers new perspectives on climatic mechanisms at the MIS 12/11 transition. The picture of the MIS 12/11 termination from marine oxygen isotope records suggests extreme changes in global ice volume^{1,23}. However, it was recently proposed that a large part of what was previously believed to be an ice-volume signal should be instead attributed to deep-water temperature changes²⁴. Furthermore, the ice build-up/isostatic mechanism for generating the 100,000-year cycle²³ has been challenged on the basis of the phase relationships with temperature, carbon dioxide and orbital eccentricity variations. Instead, the memory effect necessary to generate the 100,000-year cycle is likely to originate from the global carbon system²⁴. From Baikal's continental perspective, the new multi-proxy evidence suggests that mid-Pleistocene glaciations MIS 14 and 12 were not as extensive and severe in Siberia as were the late Pleistocene glacials. Although

clearly showing a strong 100,000-year frequency component in palaeoclimate records^{10,12}, Baikal data in the same time indicate a lack of continental ice build-up in Siberia during MIS 12 as compared with late Pleistocene. On the other hand, Baikal proxies reveal that the MIS 12/11 transition was preceded by what appears to be the longest period lacking climatic extremes on the Eurasian continent during the mid-Pleistocene. For about 110,000 years, from MIS 15a to MIS 13, high terrestrial productivity and relatively stable landscapes persisted in continental Asia. Magnetic susceptibility records of Chinese loess/soil sequences also indicate intense weathering and pedogenesis during the prolonged climatic optimum of the MIS 15–13 interval²⁵. At a time of weakened external Milankovitch insolation forcing, this long-term continental stability might have been an important component of a 100,000-year cycle climatic mechanism^{23,24}, responsible for the notable amplitude of the MIS 12/11 transition in global palaeoclimate proxy records.

If none of the above implications were to be confirmed by future studies, the Lake Baikal record still demonstrates a pronounced decoupling from the Elsterian glacial maximum of MIS 12 in Europe^{26,27}. The evidence from continental interior Asia that we report here indicates that if the marked global ice accumulation did in fact take place during MIS 12, this ice growth occurred through a mechanism different from the late Pleistocene glacial inception in the Northern Hemisphere. □

Methods

Diatom abundances were quantitatively measured in permanent smear slides²¹. Diatom valves were counted by light microscopy with a magnification of 1,000 × along transects in one of the aliquot drops using the average of 50 or 200 fields of view, depending upon diatom concentration. Diatom specimens were examined and photographed by oil immersion light microscopy, using an Ergaval microscope with a 100 × lens (numerical aperture, 1.25). For scanning electron microscopy, specimens were sputter coated with gold and observed using a JEOL (JSM-35C) scanning electron microscope.

Biogenic silica was measured using the standard wet alkaline extraction method²⁸. Measurements of magnetic susceptibility were made on whole cores at 3-cm intervals with a Bartington Instruments pass-through loop sensor operating at a low frequency (0.565 kHz) generating an alternating field of 8 μT.

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- Howard, W. R. A warm future in the past. *Nature* **388**, 418–419 (1997).
- Oppo, D. W., Fairbanks, R. G. & Gordon, A. L. Late Pleistocene Southern Ocean δ¹³C variability. *Paleoceanography* **5**, 43–54 (1990).
- Rohling, E. J. *et al.* Magnitudes of sea-level lowstands of the past 500,000 years. *Nature* **394**, 162–165 (1998).
- Hearty, P. J., Kindler, P., Cheng, H. & Edwards, R. L. A +20m middle Pleistocene sea-level highstand (Bermuda and the Bahamas) due to partial collapse of Antarctic ice. *Geology* **27**, 375–378 (1999).
- Berger, A. L. & Loutre, M.-F. Climate 400 kyr ago, a key to the future. *Eos* (Abstr.) **80**, F555 (1999).
- Ruddiman, W. F., McIntyre, A., Nieveler-Hunt, V. & Durazzi, J. T. Oceanic evidence for the mechanism of rapid northern hemisphere glaciation. *Quat. Res.* **13**, 33–64 (1980).
- Kukla, G. & Gavin, J. in *Start of a Glacial* (eds Kukla, G. & Went, E.) 307–339 (Springer, New York, 1991).
- Short, D. A. *et al.* Filtering of Milankovitch cycles by Earth's geography. *Quat. Res.* **35**, 157–173 (1991).
- Prokopenko, A. A. *et al.* Biogenic silica record of Lake Baikal response to climatic forcing during the Brunhes chron. *Quat. Res.* **55**, 123–132 (2001).
- Williams, D. F. *et al.* Lake Baikal record of continental response to orbital insolation during the past 5 million years. *Science* **278**, 1114–1117 (1997).
- BDP Members Continuous paleoclimate record of last 5 Ma from Lake Baikal. *Eos* **78**, 597–604 (1997).
- Colman, S. M. *et al.* Continental climate response to orbital forcing from biogenic silica records in Lake Baikal. *Nature* **378**, 769–771 (1995).
- Karabanov, E. B., Prokopenko, A. A., Williams, D. F. & Colman, S. M. Evidence from Lake Baikal for Siberian glaciation during oxygen-isotope substage 5d. *Quat. Res.* **50**, 46–55 (1998).
- Prokopenko, A. A., Williams, D. F., Karabanov, E. B. & Khursevich, G. K. Response of Lake Baikal ecosystem to climate forcing and pCO₂ change over the last glacial/interglacial transition. *Earth Planet. Sci. Lett.* **172**, 239–253 (1999).
- Karabanov, E. B., Prokopenko, A. A., Williams, D. F. & Khursevich, G. K. Evidence for mid-Eemian cooling in continental climatic record from Lake Baikal. *J. Paleolimnol.* **23**, 365–371 (2000).
- Peck, J. A., King, J. W., Colman, S. M. & Kravchinsky, V. A. A rock-magnetic record from Lake Baikal, Siberia: Evidence for Late Quaternary climate change. *Earth Planet. Sci. Lett.* **122**, 221–238 (1994).
- BDP Members The new BDP-98 600-m drill core from Lake Baikal: a key Late Cenozoic sedimentary section in continental Asia. *Quat. Int.* **80–81**, 19–36 (2001).
- Kuzmin, M. I. *et al.* Sedimentation processes and new age constraints on rifting stages in Lake Baikal: results of deep-water drilling. *Int. J. Earth Sci.* **89**, 183–192 (2000).
- Prokopenko, A. A. The link between tectonic and paleoclimatic events at 2.8–2.5 Ma BP in the Lake Baikal region. *Quat. Int.* **80–81**, 37–46 (2001).
- Rasskazov, S. V., Logatchev, N. A., Brandt, I. S., Brandt, S. B. & Ivanov, A. V. *Geochronology and Geodynamics in the Late Cenozoic (South Siberia - South and East Asia)* (Nauka, Novosibirsk, 2000).
- Khursevich, G. K. *et al.* Insolation regime in Siberia as a major factor controlling diatom production in

- Lake Baikal during the past 800,000 years. *Quat. Int.* **80–81**, 47–58 (2001).
- Thompson, R. & Oldfield, F. *Environmental Magnetism* (Allen and Unwin, London, 1986).
- Imbrie, J. *et al.* On the structure and origin of major glaciation cycles 2. The origin of 100,000-year cycle. *Paleoceanography* **8**, 699–735 (1993).
- Shackleton, N. J. The 100,000-year ice-age cycle identified and found to lag temperature, carbon dioxide and orbital eccentricity. *Science* **289**, 1897–1902 (2001).
- Kukla, G. & Cilek, V. Plio-Pleistocene megacycles: record of climate and tectonics. *Paleogeogr. Paleoclimatol. Paleocol.* **120**, 171–194 (1996).
- Urban, B. in *Start of a Glacial* (eds Kukla, G. & Went, E.) 37–50 (Springer, New York, 1991).
- Ehlers, J. & Linke, G. The origin of deep buried channels of Elsterian age in northwest Germany. *J. Quat. Sci.* **4**, 255–265 (1989).
- Mortlock, R. A. & Froelich, P. N. A simple method for the rapid determination of biogenic opal in pelagic marine sediments. *Deep-Sea Res.* **36**, 1415–1426 (1989).
- Laskar, J., Joutel, F. & Boudin, F. Orbital, precessional and insolation quantities for the Earth from –20 Myr to +10 Myr. *Astron. Astrophys.* **270**, 522–533 (1993).

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Mineralogy of the mid-ocean-ridge basalt source from neodymium isotopic composition of abyssal peridotites

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Inferring the melting process at mid-ocean ridges, and the physical conditions under which melting takes place, usually relies on the assumption of compositional similarity between all mid-ocean-ridge basalt sources^{1–4}. Models of mantle melting therefore tend to be restricted to those that consider the presence of only one lithology in the mantle, peridotite. Evidence from xenoliths and peridotite massifs show that after peridotite, pyroxenite and eclogite are the most abundant rock types in the mantle. But at mid-ocean ridges, where most of the melting takes place, and in ophiolites, pyroxenite is rarely found. Here we present neodymium isotopic compositions of abyssal peridotites to investigate whether peridotite can indeed be the sole source for mid-ocean-ridge basalts. By comparing the isotopic compositions of basalts and peridotites at two segments of the south-west Indian ridge, we show that a component other than peridotite is required to explain the low end of the ¹⁴³Nd/¹⁴⁴Nd variations of the basalts. This component is likely to have a lower melting temperature than peridotite, such as pyroxenite or eclogite, which could explain why it is not observed at mid-ocean ridges.

The interpretations of regular variations in major-element abun-

dances with axial ridge depth, thought to reflect a higher extent of melting and deeper initiation of melting beneath shallow ridges⁵, are only valid if the mantle source is chemically and mineralogically homogeneous. Similarly, observed correlations between $^{230}\text{Th}/^{238}\text{U}$ ratios or fractionations of the Lu–Hf and Sm–Nd system and ridge depth only bear directly on the physics of the melting process if mantle composition does not vary systematically with ridge depth^{6,7}. Chemical or mineralogical homogeneity of the source is not required in the strictest sense, but systematic mineralogical or compositional source variations with ridge depth, or with chemical parameters (as, for example, the ridge approaches a hotspot), would undermine the basis for the accepted interpretations of the correlations.

The presence of garnet-bearing mafic compositions with enriched signatures in the mantle has long been discussed^{8–12}. It is also debated whether pyroxenitic material in the mantle may account for residual garnet effects inferred from fractionation of the Lu–Hf system or ^{230}Th excesses, without requiring that melting occur within the stability field of garnet lherzolite (pressure $P \geq 2.3$ GPa)^{13,14}. Garnet-bearing eclogite or pyroxenite blobs or veins in the mid-ocean-ridge basalt (MORB) mantle would probably be associated with plume material, and thus would tend to correlate with the degree of ‘enrichment’ in the MORB mantle. Plume-affected ridge segments could have an increased chemical and isotopic enrichment of the source, owing to an increased presence of garnet pyroxenite in the source.

Most of our inferences of the MORB source are derived from the basalts. However, abyssal peridotites (AP) show chemical variations that are complementary to the basalt chemistry, and AP are the residuals of melting of a MORB source^{15–17}. In general, AP from ridge segments that are hotspot-influenced are more depleted, in terms of both chemistry and mineralogy, than AP from ridge segments that produce normal or N-type MORB only. The chemical and modal variations in AP (and complementary variations in Na_2O in associated basalts) are interpreted as variations in the degree of melting, with higher degrees of melting characterizing the sub-ridge mantle as the ridge falls under the influence of a hotspot^{15,18}. However, the co-variations of AP, MORB, ridge depth, and proximity to mantle hotspots do not account for much more than 50% of the variability of the geochemical parameters, leaving considerable room for local and regional variations in mantle composition and the dynamics of melting. We have determined the major- and trace-element content and Sr–Nd isotopic composition of clinopyroxenes (cpxs) in AP at two segments of the southwest Indian ridge (SWIR) to investigate whether the basalts can be generated solely from the AP or its AP parent before melting. In general AP are heavily serpentinized, but cpxs are most resistant to lower temperature alteration and their characteristics reflect the melting process¹⁶. Only six Nd-isotope data for AP are available¹⁹ because the severity of alteration and the low concentrations make the analysis difficult. As in previous studies, we have focused on the cpxs and present isotope data for 23 new samples.

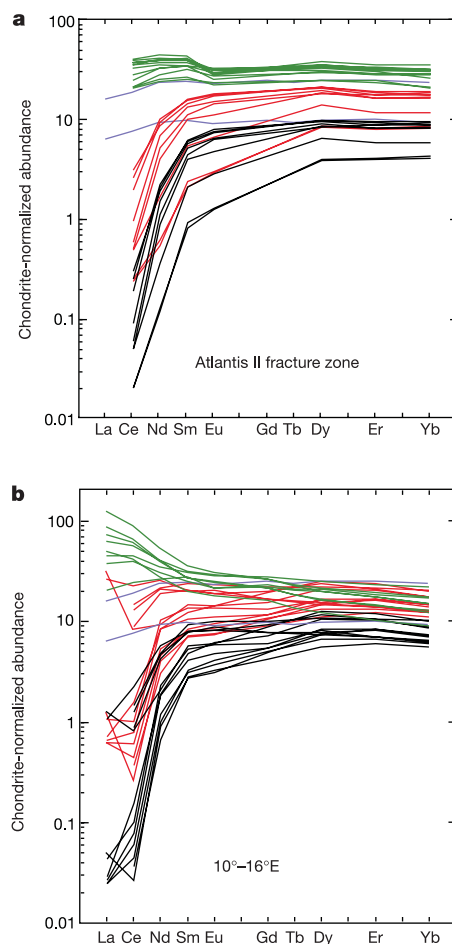


Figure 1 Chondrite-normalized REE patterns for clinopyroxenes from abyssal peridotites. Black, measured patterns in clinopyroxene (cpx); data are from this study and elsewhere^{16,17}. Red, patterns from melts in equilibrium with cpx using 1.2 GPa partition

data²⁶. Two patterns in purple indicate the field for least fractionated N-type MORB²⁷. Green, REE patterns for the ridge basalts at that segment; data are from refs 9, 20, 28, 29.

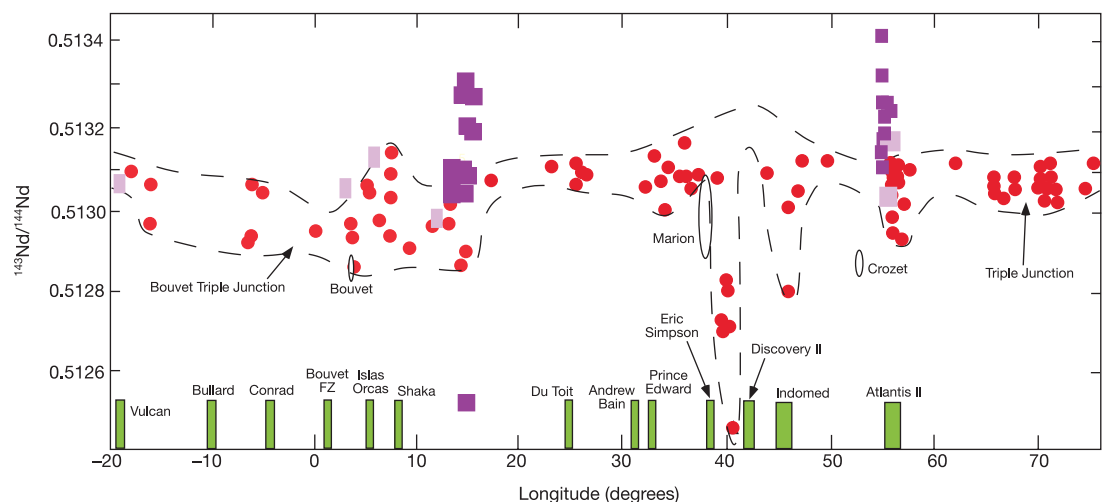


Figure 2 Nd isotopic variations in basalts and peridotites along the SWIR. Red circles, basalt data from refs 20, 28–30, and unpublished data of J. E. Snow for the AII FZ. Light purple squares, Nd isotopic compositions from cpxs in abyssal peridotites from the Snow

et al. study¹⁹. Dark purple squares, Nd isotopic composition of cpx in abyssal peridotites from this study.

We concentrated on two localities: the Atlantis II fracture zone (AII FZ) and the 10°–16° E super-segment of the SWIR. At the AII FZ (~2,300 km from the Marion hotspot), the basalts from segments on either side of this fracture zone are typical N-type MORB with depleted Nd isotopic characteristics and depleted light rare-earth-element (LREE) patterns. In contrast, the 10°–16° E segment (~600 km from the Bouvet hotspot) was chosen because here occur some of the most enriched MORB with strongly LREE enriched patterns ($La_N/Yb_N = 3.4\text{--}7.8$, where subscript N means chondrite-normalized abundance) and K_2O contents up to 1.77 wt%. The trace-element and isotope chemistry of the 10°–16° E MORB represents a mixed source where the enriched component is thought to be the easternmost expression of the Bouvet plume²⁰. The 10°–16° E segment is characterized by extreme oblique spreading (56° from orthogonal, with no transform offsets), resulting in the slowest known effective spreading rate (~4.2 mm yr⁻¹ half rate) and thin

crust (dredging and geophysical mapping show that little basaltic crust has formed over large regions²¹). On the basis of the major and trace elements in the basalts, the degree of melting at this 10°–16° E segment is significantly lower than at the AII FZ or anywhere else along the SWIR^{21,22}.

The cpxs of the AII FZ AP show strongly LREE depleted patterns, and the cpxs are too depleted in LREE to be in direct equilibrium with MORB-type melts (see Fig. 1a). However, these AP can be related to MORB through a fractional melting model¹⁶. The major-element and other trace-element chemistry (like Ti/Zr; see Table 1) confirm the rare-earth-element (REE) evidence, and show that these AP are residual after melting. The cpxs of AP from the 10°–16° E segment are more variable in composition (see Fig. 1b). In addition to cpx with strongly LREE depleted patterns, some peridotites show REE patterns that can be in equilibrium with N-type MORB melts. The major elements confirm the trace elements,

Table 1 Data for clinopyroxenes from abyssal peridotites

	⁸⁷ Sr/ ⁸⁶ Sr*	¹⁴³ Nd/ ¹⁴⁴ Nd*	Nd	Ti	Sm/Nd	Ti/Zr	Ce/Yb	Na ₂ O
Atlantis II FZ								
RC27-9 35-80	0.703447 ± 25	0.513121 ± 35	0.05	572	2.17	3,503	0.021	0.13
RC 27-9 25-138	0.704393 ± 125	0.513184 ± 11	1.94	2,110	0.55	313	0.241	1.12
RC27-9 30-44	0.703306 ± 25	0.513231 ± 15	0.08	518	1.67	1,396	0.006	0.12
RC27-9 30-44 d.		0.513228 ± 10						
RC27-9 34-62	0.704453 ± 12	0.513077 ± 43	0.56	1,104	0.88	652	0.066	0.58
RC27-9 18-51		0.513253 ± 11	0.17	661	1.43	1,552	0.014	0.30
RC27-9 6-3	0.704844 ± 66	0.513409 ± 13	0.47	1,535	1.22	662	0.034	0.38
RC27-9 35-49	0.703046 ± 13	0.513169 ± 38	0.07	627	2.01	2,337	0.021	0.097
RC27-9 6-8	0.702703 ± 56	0.513143 ± 29	0.57	1,863	1.35	1,694	0.016	0.84
RC27-9 30-10	0.702534 ± 19	0.513232 ± 12	0.66	944	0.67	640	0.074	0.56
RC27-9 34-56	0.702733 ± 39	0.513202 ± 12	0.04	537	2.83	2,731	0.727	
RC27-9 18-46	0.702685 ± 29	0.513316 ± 14	0.50	1,090	0.85	795	0.035	0.53
RC27-9 18-46 d.	0.702692 ± 13							
10–16° E								
KN162-9 42-14	0.703407 ± 55	0.513081 ± 26	0.40	1,132	1.01	1,234	0.016	
KN162-9 47-8	0.703185 ± 22	0.513099 ± 48	0.45	1,434	1.06	788	0.022	0.65
KN162-9 47-9	0.702981 ± 8	0.512502 ± 10	3.27	529	0.24	81	9.509	0.83
KN162-9 50-19	0.703013 ± 14	0.513273 ± 11	0.84	1,964	0.85	776	0.032	0.66
KN162-9 55-21		0.513206 ± 13	0.93	1,594	0.91	621	0.308	
KN162-9 59-4	0.705261 ± 11	0.513307 ± 9	1.03	1,167	0.76	321	0.058	0.68
KN162-9 59-5		0.513271 ± 12	0.53	1,119	0.85	736	0.037	
PS86-4-121	0.705637 ± 13	0.513185 ± 14	2.57	2,481	0.46	228	0.950	0.40
PS86-6-26	0.702969 ± 13	0.513031 ± 10	0.83	1,326	0.73	534	0.094	0.78
PS86-6-40	0.702554 ± 9	0.513194 ± 11	2.82	2,445	0.61	201	0.295	
PS86-6-38	0.702598 ± 13	0.513036 ± 10	2.57	2,092	0.58	207	0.326	
PS86-6-39	0.702730 ± 14	0.513050 ± 19	2.97	1,985	0.62	145	0.405	

* Isotopic compositions are fractionation-corrected using an ⁸⁶Sr/⁸⁶Sr ratio of 0.1194 and an ¹⁴⁶Nd/¹⁴⁴Nd ratio of 0.7219. Data are reported relative to an ⁸⁷Sr/⁸⁶Sr ratio of 0.708000 for the E&A standard, and an ¹⁴³Nd/¹⁴⁴Nd ratio of 0.511850 for the La Jolla standard. d., duplicate.

and do not show the extreme depletions observed at the AII FZ. However, the calculated melts in equilibrium with the less depleted AP have REE patterns that are more depleted than ridge basalts from this segment. Thus direct equilibrium between basalt and peridotite does not occur at this segment either.

At both locations, the range of isotopic compositions of the basalts and the AP cpx only partly overlap, and the lower $^{143}\text{Nd}/^{144}\text{Nd}$ ratios observed in the basalts are missing in the peridotites, and $^{143}\text{Nd}/^{144}\text{Nd}$ ratios for cpx extend to higher ratios than those from basalts (Fig. 2). One outlier at the 10° – 16° E segment with depleted Sr, but enriched Nd isotopic characteristics is excluded. This sample is in $^{143}\text{Nd}/^{144}\text{Nd}$ – $^{87}\text{Sr}/^{86}\text{Sr}$ space at such a location that it clearly cannot contribute significantly to the MORB magmatism. Although harder to interpret, the $^{87}\text{Sr}/^{86}\text{Sr}$ ratios, which are maximum values, confirm the Nd data in that the $^{87}\text{Sr}/^{86}\text{Sr}$ range in the cpx extends to lower ratios than the basalts, indicating that these peridotites represent a mantle component that, on average, is more depleted than the source for MORB. The lack of correlation with major elements such as Ti or Na shows that late-stage melt infiltration is not responsible for the Nd isotope variations in the peridotites.

Snow *et al.*¹⁹ reported Nd isotope analyses of AP (see also Fig. 2) that were within the range for MORB, and they concluded that the AP were representative of the MORB source. On closer inspection, their data show that, except for one sample at the AII FZ, all $^{143}\text{Nd}/^{144}\text{Nd}$ ratios plot at the most depleted end of the isotopic range for the 'local' basalts. The sample at the AII FZ with the lowest $^{143}\text{Nd}/^{144}\text{Nd}$ is from a veined peridotite, and interpreted to represent late-stage melt infiltration¹⁹. Thus the peridotite cpx data straddle the depleted end of the range for MORB, and extend to yet more depleted compositions. At any location, the least depleted end of the spectrum displayed by ridge basalts is not observed in the peridotites. A simple *t*-test shows that at both locations of our study the populations of AP and basalt Nd isotopic compositions are statistically different at the 99.9% confidence level, even if the data set is biased by the error of the analysis (that is, basalt $^{143}\text{Nd}/^{144}\text{Nd} + 2\sigma$, AP $^{143}\text{Nd}/^{144}\text{Nd} - 2\sigma$).

As the MORB source is thought to be the result of an ancient depletion, one could expect that a heterogeneous MORB source would display some degree of correlation between the $^{143}\text{Nd}/^{144}\text{Nd}$ and Sm/Nd ratios. The Nd isotopic compositions contributing to the basalts are then weighted by both the Nd concentration and the degree of melting. Potentially, a less depleted peridotite could be volumetrically less abundant and still contribute more Nd to the basalt than depleted peridotite. For these two ridge segments, however, there is no correlation between concentration and isotopic composition. In addition, the isotopic composition of the peridotite can be used to estimate the Nd concentration of the peridotites before melting. Assuming a 2-Gyr age for the MORB source and using a simple melting model, we can calculate an Nd concentration for each peridotite and a concentration-weighted average isotopic composition for the depleted component in the MORB source at each ridge segment. If the basalt source is a mixture between this depleted, observed, component and a less depleted component (which is missing in our data set), and if the less depleted component is assumed to be bulk Earth, then for the AII FZ 12% of such a bulk Earth component is needed to explain the discrepancy between peridotites and basalts. For the 10 – 16° E segment, 20% of a bulk Earth component is required. We have selected the samples for isotope analysis with the aim of covering the complete spectrum of mineralogical and chemical variability. The 11 peridotite samples from the AII FZ are representative of the 20 samples from the studies of Johnson *et al.*^{16,17}, and it seems unlikely that a significant part of the spectrum, required to balance the present data set, was missed. The 12 AP from the 10° – 16° E segment were chosen from a suite of 17 peridotite dredges distributed over the entire segment, and are representative of the 'local' mantle. The range in

REE patterns observed at this segment is almost as large as the entire range observed for AP, thus it is again unlikely that a significant part of the peridotite spectrum was missed. We conclude that the Nd isotope variations in the peridotites are representative of the AP at the ridge.

It could be argued that comparison of peridotite with present-day ridge basalts is not correct. The present ridge basalts are derived from mantle still ascending and not yet at the sea floor. The basalts derived from the analysed peridotites form several-million-year-old oceanic crust now removed from the ridge. Temporal variations in the MORB source could be invoked, and used to argue that the present-day basalt source is a less depleted peridotite than those analysed. However, the peridotites are at the high $^{143}\text{Nd}/^{144}\text{Nd}$ end at any location, implying that along the length of the SWIR the present-day mantle is significantly more enriched than several million years ago. We do not consider this likely, however, because comparisons of old AP at fracture zones with spatially associated basalts have found a good correlation between the two, suggesting no large changes in the source on the timescale of a few million years^{15,16}. This hypothesis could be further tested, though, by off axis sampling of the oceanic crust.

An alternative, and more likely, hypothesis is that the enriched component is a non-peridotitic component, but is a component with a lower melting point than peridotite, such as pyroxenite or eclogite. The possible existence of eclogite or pyroxenite in the mantle has been argued for by many workers (see above). The bimodality of the basalt compositions at the 10 – 16° E area has also been used to argue for a bimodal source (peridotite and pyroxenite)²⁰. Our study shows, to our knowledge for the first time, that the isotopic variations in the ridge basalt require the presence of a component not observed in the peridotites at the ridge. This can be accomplished by including a component with a lower solidus than peridotite, and the most likely candidates are pyroxenite or eclogite. This component is largely exhausted before the melting of the peridotite stops, and is therefore not observed on the ocean floor. At present the amount of pyroxenite or eclogite in the source of the SWIR basalts is still uncertain. As these materials are more enriched in trace elements compared to peridotite, a small amount could have large influence on the isotopic composition.

Indian Ocean basalts have isotopic characteristics that are significantly different from those for ridge basalts from the Pacific and Atlantic oceans. It remains to be determined whether such a non-peridotitic enriched component is restricted to the SWIR (or Indian Ocean), or whether it also occurs at other locations. Some evidence that the pyroxenite-veined mantle is widespread comes from observations from intra-transform spreading centres and other locations where basalts are produced from a mantle source inferred to have previously melted. For example, MORB from a small intra-transform rift in the Garrett fracture zone, southern East Pacific Rise (EPR), are more depleted in terms of Nd, Sr and Pb isotope signatures than are MORB from the adjacent EPR segments²³. 'Zero age' MORB from the Australian–Antarctic discordance at the Indian–Pacific mantle boundary region, interpreted to derive from previously depleted mantle, has lower $^{206}\text{Pb}/^{204}\text{Pb}$ ratios, lower Sr content and higher Nd isotope ratios²⁴. These observations support the idea that there is a lower temperature melting component in the MORB source that is extracted preferentially during mantle melting. However, those melting models that explain ridge basalt chemistry solely on the basis of peridotite melting should be treated with caution, especially for the case of the SWIR. □

Methods

We determined major-element content *in situ* by electron microprobe²⁵ and trace elements *in situ* by ion microprobe¹⁶. For isotope analyses, samples were crushed and sieved to obtain a 200–500- μm fraction. This fraction was leached with hydrogen peroxide, which removed a large part of the manganese and iron oxides, providing a 'cleaner' fraction for

magnetic separation. A cpx-rich fraction was produced using a Frantz isodynamic separator; this fraction was hand-picked under a binocular microscope to obtain a 'pure' cpx fraction. Nd concentrations in cpx are as low as 50 p.p.b., and in general 100 mg of cpx was hand-picked. But this hand-picked fraction still contains some alteration products (distributed in a pattern similar to the crystal structure). Leaching experiments on the cpx separates found that hydrochloric acid at 100 °C removes this alteration to the point that the Nd isotopic composition is reproducible after only few hours of leaching. See also Snow *et al.*¹⁹. The Nd concentration in sea water and hydrothermal waters is low compared to those in cpx, and the effect of alteration on ¹⁴³Nd/¹⁴⁴Nd ratio is limited. However, the Sr content of sea water and hydrothermal fluids is much higher, and effects of alteration on the ⁸⁷Sr/⁸⁶Sr ratio are harder to remove. Cpxs from abyssal peridotites dissolve even if leaching is done with hydrochloric acid only, and only a limited amount of leaching can be done to attempt to obtain a pristine Sr isotopic composition. The cpx fractions were leached with sub-boiling double distilled 6 M hydrochloric acid at 100 °C for 24 h. This sometimes resulted in the removal of most secondary Sr. In almost all cases, leached cpxs have lower ⁸⁷Sr/⁸⁶Sr ratios than the leachates, and the reported values are upper limits for these samples. Dissolution and separation chemistry was performed using standard techniques.

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- McKenzie, D. & O'Nions, R. K. Partial melt distributions from inversion of rare earth element concentrations. *J. Petrol.* **32**, 1021–1091 (1991).
- Langmuir, C. H., Klein, E. M. & Plank, T. in *Mantle Flow and Melt Generation at Mid-ocean Ridges* (eds Morgan, J. P., Blackman, D. K. & Sinton, J. M.) 183–280 (American Geophysical Union, Washington DC, 1992).
- Spiegelman, M. & Elliott, T. Consequences of melt transport for uranium series disequilibrium in young lavas. *Earth Planet. Sci. Lett.* **118**, 1–20 (1993).
- Salters, V. J. M. & Hart, S. R. The Hf-paradox, and the role of garnet in the MORB source. *Nature* **342**, 420–422 (1989).
- Klein, E. M. & Langmuir, C. H. Global correlations of ocean ridge basalt chemistry with axial depth and crustal thickness. *J. Geophys. Res.* **92**, 8089–8115 (1987).
- Bourdon, B., Zindler, A., Langmuir, C. H. & Elliott, T. ²³⁰Th–²³⁸U systematics in MORB: a global perspective. *Nature* **384**, 231–235 (1996).
- Salters, V. J. M. The generation of mid-ocean ridge basalts from the Hf and Nd isotope perspective. *Earth Planet. Sci. Lett.* **141**, 109–123 (1996).
- Hanson, G. N. Geochemical evolution of the suboceanic mantle. *J. Geol. Soc. Lond.* **134**, 1–19 (1977).
- le Roex, A. P. *et al.* Geochemistry, mineralogy and petrogenesis of lavas erupted along the southwest Indian Ridge between the Bouvet Triple Junction and 11 degrees east. *J. Petrol.* **24**, 267–318 (1983).
- Zindler, A., Staudigel, H. & Batizza, R. Isotope and trace element geochemistry of young Pacific seamounts: implications for the scale of upper mantle heterogeneity. *Earth Planet. Sci. Lett.* **70**, 175–195 (1984).
- Sleep, N. H. Tapping of magmas from ubiquitous mantle heterogeneities: An alternative to mantle plumes. *J. Geophys. Res.* **89**, 10029–10041 (1984).
- Allègre, C. J. & Turcotte, D. L. Implications of a two component marble-cake mantle. *Nature* **323**, 123–127 (1986).
- Hirschmann, M. M. & Stolper, E. M. A possible role for garnet pyroxenite in the origin of the "garnet signature" in MORB. *Contrib. Mineral. Petrol.* **124**, 185–208 (1996).
- Stracke, A., Salters, V. J. M. & Sims, K. W. W. Assessing the presence of pyroxenite in the source of Hawaiian basalts: hafnium-neodymium-thorium isotope evidence. *Geochim. Geophys. Geosyst.* **1**, 1999GC000013 (2000).
- Dick, H. J. B., Fisher, R. L. & Bryan, W. B. Mineralogical variability of the uppermost mantle along mid-ocean ridges. *Earth Planet. Sci. Lett.* **69**, 88–106 (1984).
- Johnson, K. T. M., Dick, H. J. B. & Shimizu, N. Melting in the oceanic upper mantle: an ion microprobe study of diopsides in abyssal peridotites. *J. Geophys. Res.* **95**, 2661–2678 (1990).
- Johnson, K. T. M. & Dick, H. J. B. Open system melting and temporal and spatial variation of peridotite and basalt at the Atlantis II fracture zone. *J. Geophys. Res.* **97**, 9219–9241 (1992).
- Hellebrand, E., Snow, J. E., Dick, H. J. B. & Hofman, A. W. Coupled major and trace elements as indicators of the extent of melting in mid-ocean-ridge peridotites. *Nature* **410**, 677–681 (2001).
- Snow, J. E., Hart, S. R. & Dick, H. J. B. Nd and Sr isotope evidence linking mid-ocean ridge basalts and abyssal peridotites. *Nature* **371**, 57–60 (1994).
- le Roex, A. P., Dick, H. J. B. & Watkins, R. T. Petrogenesis of anomalous K-enriched MORB from the Southwest Indian Ridge: 11°53' E to 14°38' E. *Contrib. Mineral. Petrol.* **110**, 253–268 (1992).
- Dick, H. J. B., Schouten, H. & Lin, J. Crustal(?) accretion during extreme oblique spreading at an ultra-slow mid-ocean ridge. *Eos* **82**, S407 (2001).
- Dick, H. J. B., Georgen, J. E., le Roex, A. P. & Lin, J. The influence of ridge geometry on mantle melting at an ultra-slow spreading ridge. *Eos* **79**, F919 (1998).
- Wendt, J. L., Regelous, M., Niu, Y., Hékinian, R. & Collerson, K. D. Geochemistry of lavas from the Garrett Transform Fault: insights into mantle heterogeneity beneath the eastern Pacific. *Earth Planet. Sci. Lett.* **173**, 271–284 (1999).
- Hanan, B. B., Blichert-Toft, J., Pyle, D. G., Christie, D. & Albarède, F. Ultra-depleted hafnium isotopes from Australian-Antarctic discordance MORB. *J. Conf. Abstr.* **5**, 478 (2000).
- Keshav, S. & Sen, G. Majoritic garnets in Hawaiian xenoliths: preliminary results. *Geophys. Res. Lett.* **28**, 3509–3512 (2001).
- Salters, V. J. M. & Longhi, J. E. Trace element partitioning during the initial stages of melting beneath ocean ridges. *Earth Planet. Sci. Lett.* **166**, 15–30 (1999).
- Sun, S.-S. & McDonough, W. F. in *Magmatism in the Ocean Basins* (eds Saunders, A. D. & Norry, M. J.) 313–345 (Geological Society, London, 1989).
- Mahoney, J. J. *et al.* Isotopic and geochemical provinces of the western Indian Ocean spreading centers. *J. Geophys. Res.* **94**, 4033–4052 (1989).
- Mahoney, J., le Roex, A. P., Peng, Z., Fisher, R. L. & Natland, J. H. Southwestern limits of Indian Ocean Ridge mantle and the origin of low ²⁰⁶Pb/²⁰⁴Pb mid-ocean ridge basalt: Isotope systematic of the Central Southwest Indian Ridge (17–50E). *J. Geophys. Res.* **97**, 19771–19790 (1992).

30. le Roex, A. P., Dick, H. J. B. & Fisher, R. L. Petrology and geochemistry of MORB from 25°E to 46°E along the southwest Indian ridge: Evidence for contrasting styles of mantle enrichment. *J. Petrol.* **30**, 947–986 (1989).

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Competing interests statement

The authors declare that they have no competing financial interests.

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An early tetrapod from 'Romer's Gap'

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The fossil record of early tetrapods has been increased recently by new finds from the Devonian period¹ and mid-late Early Carboniferous period². Despite this, understanding of tetrapod evolution has been hampered by a 20-million-year gap ('Romer's Gap'³) that covers the crucial, early period when many key features of terrestrial tetrapods were acquired. Here I describe the only articulated skeleton of a tetrapod, *Pederpes*, yet found from the Tournaisian epoch (354–344 million years ago (Myr)). The new taxon includes a pes with five robust digits, but a very small, possibly supernumerary digit preserved on the manus suggests the presence of polydactyly. Polydactylous early tetrapods may have survived beyond the end of the Devonian and pentadactyly cannot be assumed for the pes. However, the pes has characteristics that distinguish it from the paddle-like feet of the Devonian forms and resembles the feet of later, more terrestrially adapted Carboniferous forms. *Pederpes* is the earliest-known tetrapod to show the beginnings of terrestrial locomotion and was at least functionally pentadactyl. With its later American sister-genus, *Whatcheeria*^{4,5}, it represents the next most primitive tetrapod clade after those of the Late Devonian, bridging the temporal, morphological and phylogenetic gaps that have hitherto separated Late Devonian and mid-Carboniferous tetrapod faunas.

Misidentified as a rhizodont fish on its discovery in 1971, recent preparation has revealed the specimen to be a tetrapod of about 650 mm in presacral length, lacking only a few parts of the skull, the tail, and some limb elements (Fig. 1a, b).

Spore analysis shows that the specimen dates from the late Tournaisian. Isolated limb and girdle elements from Horton Bluff in Canada are the only other tetrapod fossils known from this part of the Early Carboniferous²; this time period is otherwise remarkable for its lack of fossils from continental deposits. The recently described *Casineria kiddi*² and the tetrapods from East Kirkton (for example, ref. 2) are Viséan, about 10 and 15 million years younger, respectively. *Pederpes* is the only Early Carboniferous tetrapod from western Scotland, extending the known geographical range of Early Carboniferous forms, but more importantly, it raises the possibility of further finds in this relatively unexplored area.

The specimen derives from the Ballagan Formation, which was laid down in a shallow-water environment, probably a lagoon or coastal flat that was subject to marked fluctuations in salinity and periodic desiccation⁶. The formation consists of pale grey, fine-grained nodular cementstones with calcareous mudstones and limestones⁷. The specimen is preserved in a clayey limestone nodule typical of a cementstone facies. Except for a few isolated rhizodont and actinopterygian scales, no other vertebrate fossils have been found in the area, so this tetrapod specimen appears to be an erratic. Fish and plant debris as well as *Spirorbis* shells are preserved in the nodule, the latter suggesting at least a marginal marine influence in the preservation of the animal.

Tetrapodomorpha Ahlberg, 1991

Whatcheeriidae fam. nov.

Pederpes finneyae gen. et sp. nov.

Etymology. *Pederpes*, Peder after Peder Aspen, its discoverer (Peder is the Norwegian form of Peter, which is Greek for rock), and *erpes* (Greek for crawler), that is, ‘rock crawler’ (*Pederpes* can also be split into Peder and *pes* (foot), that is, ‘rock foot’). *finneyae*, after S. M. Finney, who prepared the specimen.

Holotype. Hunterian Museum, Glasgow, GLAHMS 100815, an almost complete articulated skeleton in a clayey limestone nodule.

Locality. Auchenreoch Glen, near Maryland Farm, 2–3 km north of Dumbarton, Scotland.

Horizon and stratigraphy. Ballagan Formation, Inverclyde Group (formerly Cementstone Group, Calciforous Sandstone Series)⁶⁷; *claviger-macra* (CM) palynozone (possibly from the lower part of this zone); Tournaisian Tn3c, Courcayan, Dinantian, Lower Carboniferous.

Age. Ivorian, Early Carboniferous (348–344 Myr).

Diagnosis of family. Derived features include: narrow, steep-sided skull with orbit deeper than its width; massive tooth on maxilla about position 5 or 6; light dermal skull ornament; dorsal branch of mandibular lateral line running along surangular; very broad interclavicle with acutely angled lateral corners.

Primitive features include: grooved, denticulated parasphenoid; closed palate with denticulated surface; supratemporal-postparietal contact, fang pairs on vomers, palatines and ectopterygoids with a row of some smaller accessory teeth on each; row of coronoid teeth nearly continuous; at least some lateral lines in tubes through bone; rachitiform vertebrae; no differentiated sacral neural arch, ilium with posterodorsal process and dorsal iliac blade.

Features of uncertain polarity include: supratemporal with deeply interdigitated suture to squamosal; small tabular with 'button' terminating in ornamented surface; steeply angled suspensorium with deeply excavated temporal notch, pronounced angle between skull table and cheek in transverse section; scapulocoracoid ossified in two portions; about 28 presacral vertebrae; trunk ribs with expanded distal flanges.

Diagnosis of genus and only known species. Whatcheeriid with the following apomorphies: trunk ribs with at least vertebrae

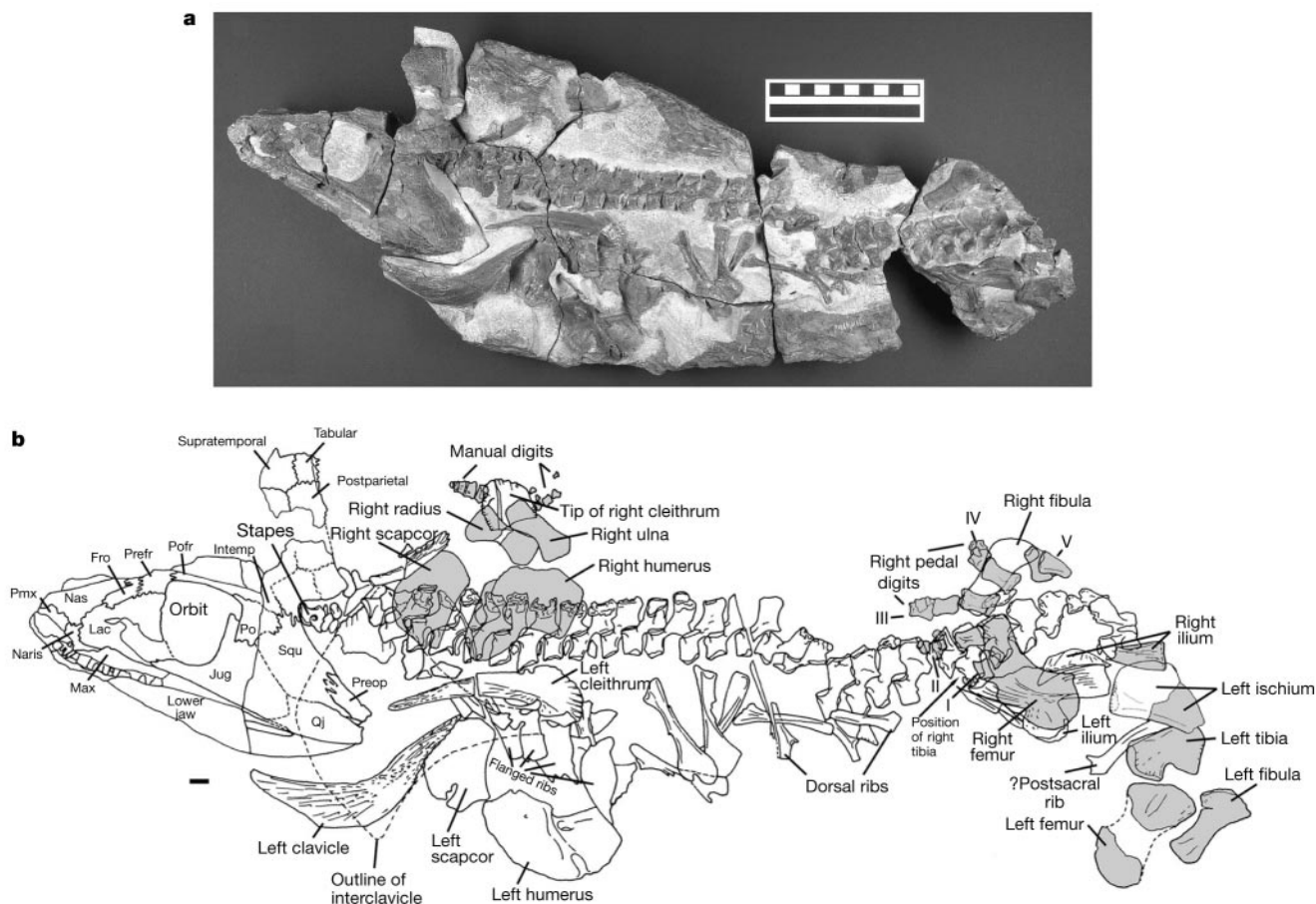


Figure 1 Holotype specimen of *Pederpes finneyae*. **a**, GLAHMS 100815 *Pederpes finneyae*, photograph of holotype and only specimen. Scale bar, 100 mm; **b**, Map of the holotype, showing elements preserved on the reverse in grey. Scale bar, 10 mm. Fro,

frontal; intemp, intertemporal; jug, jugal; lac, lacrimal; max, maxilla; nas, nasal; pmx, premaxilla; po, postorbital; pofr, postfrontal; prefr, prefrontal; preop, preopercular; qj, quadratojugal; scapcor, scapulocoracoid.

numbers 4–9 bearing accessory processes and/or foramina at dorsal edge of acutely triangular terminal expansions and about numbers 10–12 with flared ends; short presacral ribs with accessory processes; minute lateral-most digit on manus; deep striations on anterior edge and anterior region of external surface of stem of clavicle and cleithrum; ovoid dorsal blade of cleithrum with fimbriated edge.

The specimen is distinguished from *Whatcheeria deltae* by many details of the cranial and postcranial skeleton listed under Appendix 1 of the Supplementary Information.

Notable anatomical features of *Pederpes* include a primitive stapes most closely resembling that of the Devonian *Acanthostega*^{8,9} in a narrow skull with a deep wide temporal notch. It unequivocally dissociates such a notch from possession of a slender sound-conducting element (Fig. 2). The humerus has a spike-like latissimus dorsi process more like that of *Baphetes*¹⁰ than *Whatcheeria* or any other tetrapod, presumably convergently derived. The ribs bear expanded triangular flanges, a feature now found in a range of the earliest tetrapods. Those of *Pederpes* most closely resemble

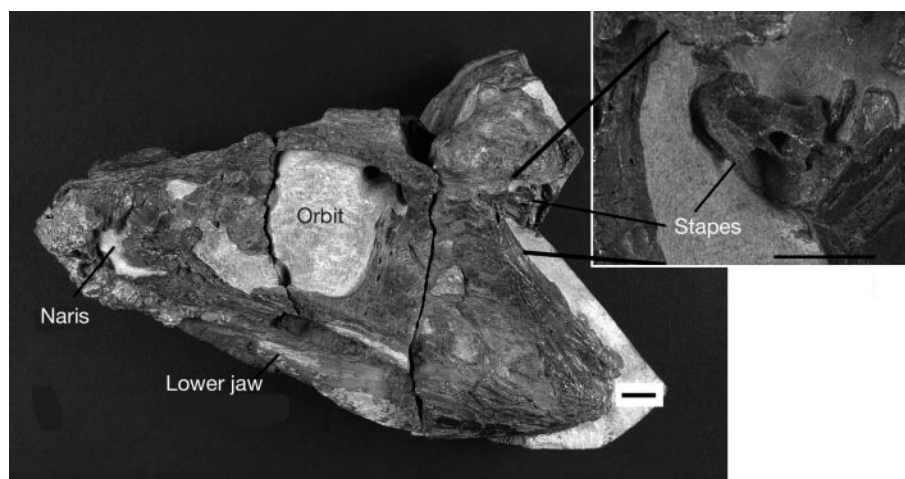


Figure 2 Close-up of the skull of *Pederpes* showing the stapes. Inset, stapes. Scale bars, 10 mm.

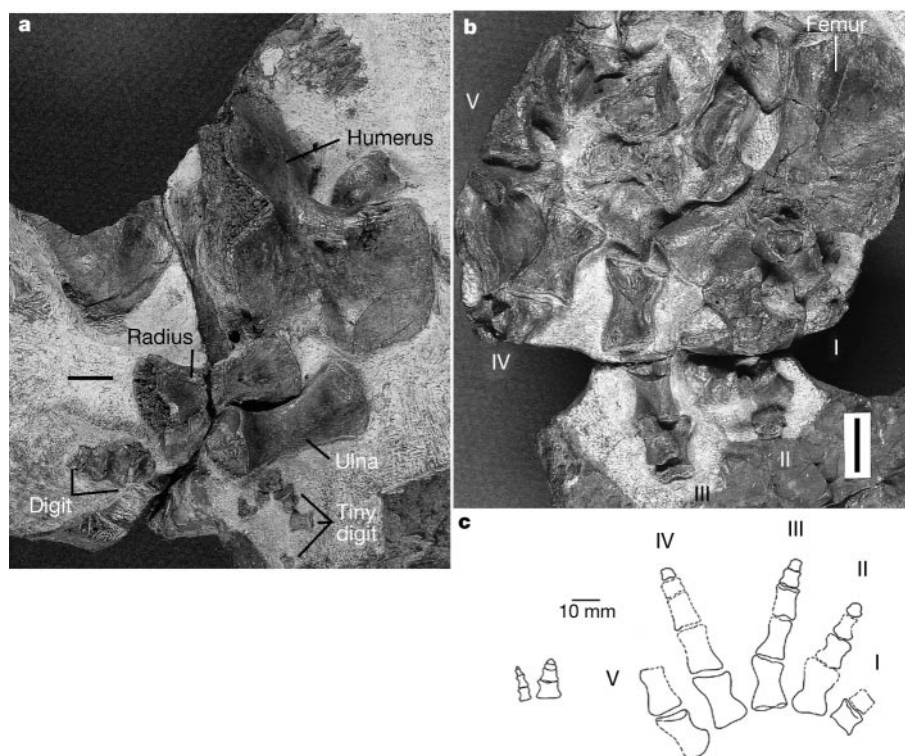


Figure 3 Manus and pes of *Pederpes*. **a**, Humerus, radius, ulna and manual digits from the right forelimb. The minute irregular elements at the base of the tiny digit might be remains of metacarpals. **b**, Pedal digits and femur from the right hindlimb, shown reversed. **c**, Reconstructions of manual (left) and pedal digits (right) of *Pederpes* to the same scale, showing tiny manual digit and asymmetrical metatarsals respectively. Scale

bar, 10 mm. In *Pederpes*, the phalanges of the tiny manual digits are about one-third the length of the pedal phalanges, whereas in *Acanthostega* and the Carboniferous forms such as *Greererpeton* and *Proterogyrinus*, manual phalanges are about one-half the length of the pedal phalanges.

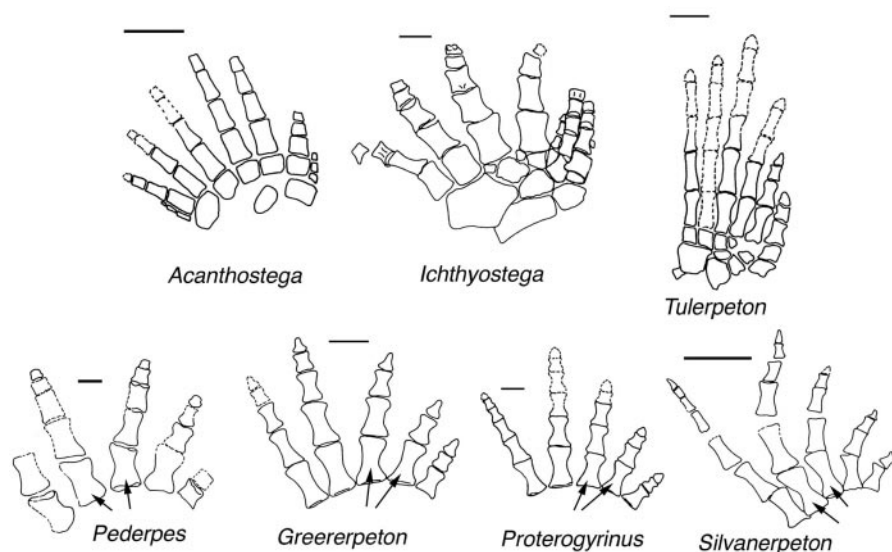


Figure 4 Reconstruction of peses of various taxa. *Pederpes*, *Greererpeton*, *Silvanerpeton* and *Proterogyrinus* show asymmetrical metatarsals (see arrows) compared with those of the Devonian forms *Acanthostega*, *Ichthyostega* and *Tulerpeton*. In *Acanthostega* and

Ichthyostega, the metatarsals are not clearly differentiated, and in *Tulerpeton*, if correctly interpreted, they are cylindrical but include some interarticulations. Scale bars, 10 mm.

those of *Ichthyostega*.

Two digits (only) are preserved on the right manus. One is short, broad and tapered, but the more remarkable is a tiny digit with three phalanges including an ungual, that presumably lay lateral-most on the manus. In its relative size, it most closely resembles the super-

numerary digits found on the limbs (especially the hindlimbs) of the polydactylous Devonian tetrapods^{11,12} (Figs 3 and 4). *Pederpes* conceivably retained a polydactylous manus, suggesting that polydactylous early tetrapods survived into the Carboniferous.

The pes of *Pederpes* preserves a complement of five digits, three of which are complete. Although there is no evidence for more than five digits (Fig. 3b, c), recent understanding of early limb evolution and the existence of the tiny manual digit preclude firm assumptions of pentadactyly in this early tetrapod. No complete manus or pes is known for *Whatcheeria*, and that animal also cannot be assumed to have been pentadactylous. However, the pes of *Pederpes* is closely comparable with those of later Carboniferous tetrapods, especially *Greererpeton*, in its proportions and numbers of phalanges (Fig. 4). It shares a feature otherwise seen only in the pentadactylous forms such as *Greererpeton*¹³, *Silvanerpeton*¹⁴ and *Proterogyrinus*¹⁵, which are assumed to be at least partially terrestrial: they have clearly distinguishable metatarsals that are bilaterally and proximodistally asymmetrical (Fig. 4). This feature may be associated with realignment of the foot from a lateral orientation as in the paddle-like limbs of Devonian forms, into an anteriorly directed stance associated with walking. Thus the pes of *Pederpes* shows an adaptation otherwise known only in pentadactylous forms, and is the earliest form to show what later becomes an established pattern of foot construction.

A phylogenetic analysis shows the whatcheerids to be the next most primitive clade of tetrapods after those of the Devonian, a position congruent with both the stratigraphic position and the morphology of the animals. As well as showing the beginnings of terrestrial locomotion, *Pederpes*, with its sister taxon *Whatcheeria*, provides a view of the preconditions leading to the tetrapod crown group, whose origins are unclear. □

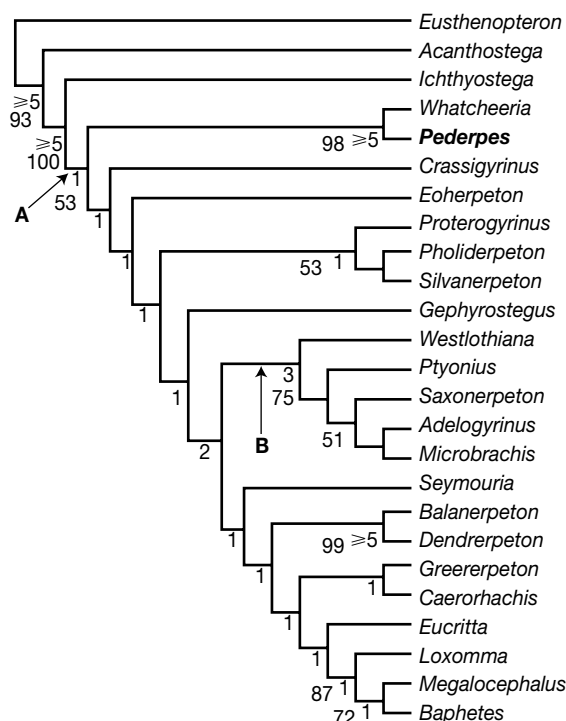


Figure 5 Single most parsimonious tree showing the whatcheerids to be the next most crownward stem tetrapod clade after the Devonian forms. Nodes A and B show possible origins of the crown group. Bremer support indices are shown for most nodes, and bootstrap values are given for each node supported by more than 50%.

Methods

A phylogenetic analysis using 25 taxa and 141 characters was carried out using PAUP 4.0b (ref. 16) and MacClade 3.04 (ref. 17). A range of early tetrapods was analysed including several 'lepospondyl' taxa and the possible stem amniote *Westlothiana*. The analysis recovered a single most parsimonious tree (MPT) with length 484, consistency index 0.393, retention index 0.561, and rescaled consistency index 0.220. Bremer support values for most nodes and bootstrap values for the best-supported nodes are shown (Fig. 5). Despite the low consistency index, a permutation tail-probability test (PTP) using the PAUP program (10,000 replicates) showed that the data matrix contains a signal

statistically significant (P -value of 0.0001) relative to randomized data, and is thus phylogenetically informative.

The single most parsimonious tree shows the whatcheeriids to be the next most primitive clade after the Devonian forms, with this node supported better than many others in the tree. This analysis produces an unexpected crown topology in some respects, although the data set was designed to explore the relationships of the basal taxa rather than of those more crownward. The three most basal nodes of the tree are robust, but many of the more crownward nodes are only weakly supported, reflecting the radically different views of early tetrapod relationships that have recently been published (compare refs 12, 18–23). According to different analyses, the crown group node could originate as deep as node A (for example, ref. 19) or as crownward as node B (for example, ref. 20); thus the whatcheeriids lie at the base of the most inclusive definition of the crown group clade. Deletion of the taxa above node B does not significantly affect the topology of the rest of the tree, but deletion of the whatcheeriids or the enigmatic genus *Caerorhachis* recovers a grouping including embolomeres plus *Gephyrostegus* and *Eoherpeton*, the traditional ‘anthracosaurs’. A reverse constraint analysis was undertaken to test how strongly the basal position of whatcheeriids is in relation to ‘other tetrapods’ apart from Devonian forms. In this test, all other tetrapods above the whatcheeriids were defined as being monophyletic. PAUP was then asked to find the shortest trees under the reverse constraint, in which ‘other tetrapods’ was not monophyletic. Nine trees four steps longer than the MPT (length 488) were recovered, with the common property of placing *Crassigyrinus* as the next most primitive taxon after the Devonian forms. Eight of these recovered a monophyletic grouping of the traditional ‘anthracosaurs’. A Templeton test reveals no statistically significant difference between the MPT and those recovered in the reverse constraint analysis. *Whatcheeria* and *Crassigyrinus* were originally described as ‘protoanthracosaurs’^{4,24}, but this may result from the presence of many primitive characters in the traditional ‘anthracosaurs’, whose relationships are in need of review.

See Appendices 1–4 of the Supplementary Information for further diagnosis of *Pederpes* (Appendix 1), source data (Appendix 2), the data matrix (Appendix 3) and the character list (Appendix 4).

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1. Clack, J. A. in *Amphibian Biology* (eds Heatwole, H. & Carroll, R. L.) 979–1029 (Surrey Beatty, Chipping Norton, Australia, 2000).
2. Clack, J. A. & Carroll, R. L. in *Amphibian Biology* (eds Heatwole, H. & Carroll, R. L.) 1030–1043 (Surrey Beatty, Chipping Norton, Australia, 2000).
3. Coates, M. I. & Clack, J. A. in *Studies on Early Vertebrates* (eds Arsenault, M., Lelièvre, H. & Janvier, P.) 373–388 (Bulletin du Muséum National d'Histoire Naturelle, Paris, 1995).
4. Lombard, R. E. & Bolt, J. R. A new primitive tetrapod, *Whatcheeria deltae*, from the Lower Carboniferous of Iowa. *Palaeontology* **38**, 471–494 (1995).
5. Bolt, J. R. & Lombard, R. E. in *Amphibian Biology* (eds Heatwole, H. & Carroll, R. L.) 1044–1052 (Surrey Beatty, Chipping Norton, Australia, 2000).
6. Paterson, I. B., Hall, I. H. S. & Stephenson, D. *British Geological Survey, Geology of the Greenock district* (Her Majesty's Stationery Office (HMSO), London, 1990).
7. Cameron, I. B. & Stephenson, D. *British Geological Survey, British Regional Geology; The Midland Valley of Scotland* (Her Majesty's Stationery Office (HMSO), London, 1985).
8. Clack, J. A. Earliest known tetrapod braincase and the evolution of the stapes and fenestra ovalis. *Nature* **369**, 392–394 (1994).
9. Clack, J. A. The neurocranium of *Acanthostega gunnari* and the evolution of the otic region in tetrapods. *Zool. J. Linn. Soc.* **122**, 61–97 (1998).
10. Milner, A. C. & Lindsay, W. Postcranial remains of *Baphetes* and their bearing on the relationships of the Baphetidae (= Loxommatidae). *Zool. J. Linn. Soc.* **122**, 211–235 (1998).
11. Coates, M. I. & Clack, J. A. Polydactyly in the earliest known tetrapod limbs. *Nature* **347**, 66–69 (1990).
12. Coates, M. I. The Devonian tetrapod *Acanthostega gunnari* Jarvik: postcranial anatomy, basal tetrapod relationships and patterns of skeletal evolution. *Trans. R. Soc. Edinb. Earth Sci.* **87**, 363–421 (1996).
13. Godfrey, S. J. The postcranial skeletal anatomy of the Carboniferous tetrapod *Greererepton burkemorani* Romer, 1969. *Phil. Trans. R. Soc. Lond. B* **323**, 75–133 (1989).
14. Clack, J. A. *Silvanerpeton miripede*, a new anthracosauroid from the Viséan of East Kirkton, West Lothian, Scotland. *Trans. R. Soc. Edinb. Earth Sci.* **84**, 369–376 (1994).
15. Holmes, R. The Carboniferous amphibian *Proterogyrinus scheeli* Romer, and the early evolution of tetrapods. *Phil. Trans. R. Soc. Lond. B* **306**, 431–524 (1984).
16. Swofford, D. L. *PAUP: Phylogenetic analysis using parsimony* Version 3.1. (distributed by the Illinois Natural History Survey, Champaign, Illinois, 1993).
17. Maddison, W. P. & Maddison, D. R. *MacClade Version 3.0 Analysis of phylogeny and character evolution* (Sinauer, Sunderland, Massachusetts, 1992).
18. Carroll, R. L. in *Studies on Early Vertebrates* (eds Arsenault, M., Lelièvre, H. & Janvier, P.) 389–445 (Bulletin du Muséum National d'Histoire Naturelle, Paris, 1995).
19. Lebedev, O. A. & Coates, M. I. The postcranial skeleton of the Devonian tetrapod *Tulerpeton curtum* Lebedev. *Zool. J. Linn. Soc.* **114**, 307–348 (1995).
20. Laurin, M. & Reisz, R. in *Amniote origins—completing the transition to land* (eds Sumida, S. & Martin, K. L.) 9–59 (Academic, London, 1997).
21. Clack, J. A. A new Lower Carboniferous tetrapod with a mélange of crown group characters. *Nature* **394**, 66–69 (1998).
22. Paton, R. L., Smithson, T. R. & Clack, J. A. An amniote-like skeleton from the Early Carboniferous of Scotland. *Nature* **398**, 508–513 (1999).
23. Anderson, J. S. The phylogenetic trunk: maximal inclusion of taxa with missing data in an analysis of the Lepsospondyli (Vertebrata, Tetrapoda). *Syst. Biol.* **50**, 170–193 (2001).
24. Panchen, A. L. On the amphibian *Crassigyrinus scoticus* Watson from the Carboniferous of Scotland. *Phil. Trans. R. Soc. Lond. B* **309**, 461–568 (1985).

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Competing interests statement

The author declares no competing financial interests.

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A host–parasite interaction rescues *Drosophila* oogenesis defects

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The cytoplasmically inherited bacterium *Wolbachia pipientis* is a widespread parasite of arthropods that manipulates the reproductive biology of its hosts, often to their detriment, in order to foster its own transmission through egg cytoplasm^{1,2}. Here we report that infection by *Wolbachia* restores fertility to *Drosophila melanogaster* mutant females prevented from making eggs by protein-coding lesions in *Sex-lethal* (*Sxl*), the master regulator of sex determination. Suppression of sterility by *Wolbachia* discriminates markedly among similar germline-specific *Sxl* alleles, and is not observed for mutations in other genes that produce similar ‘tumorous ovary’ phenotypes, including one that blocks *Sxl* germline expression. This allele and gene specificity indicates that suppression probably results from a specific interaction with *Sxl* protein, rather than from a bypass of the normal germline requirement for this developmental regulator or from an effect on *Sxl* expression. The *Sxl*–*Wolbachia* interaction provides a rare opportunity to explore host–parasite relationships at the molecular level in a model insect. Furthermore, demonstration that a parasite infection can counteract the deleterious effects of mutations in host genes illustrates how hosts might become dependent on parasites.

Because *Wolbachia* depends on obligate maternal transmission, it manipulates the reproduction of its host to increase the number of infected females³. Reproductive phenotypes caused by *Wolbachia* infection include parthenogenesis⁴, feminization⁵, male killing⁶, and cytoplasmic incompatibility (inviability of offspring from a mating of infected males with uninfected females)⁷. These effects can skew sex ratios and may even promote speciation⁸. Although *Wolbachia* infection generally has no obvious benefit to the host, the parasitic wasp *Asobara tabida* can be considered an exception. In a remarkable natural parallel to the laboratory phenomenon described here, *Asobara* requires *Wolbachia* infection for oogenesis⁹. Little is known about the mechanisms *Wolbachia* uses to affect reproduction, in part because the host species that display strong phenotypes have not been readily amenable to genetic analysis. Although infection in *D. melanogaster* has been reported to cause low levels of cytoplasmic incompatibility¹⁰ and a shortening of adult life span¹¹, suppression of mutant *Sex-lethal* alleles that we report here represents a robust phenotype for *Wolbachia* infection in this model organism.

Sex-lethal (*Sxl*) is an X-linked, female-specific master switch gene that controls somatic sex determination and the vital process of

Table 1 *Wolbachia* infection rescues an oogenesis defect of *Sxl^{f4}* mutant females

Genotype†	<i>Wolbachia</i> source	Females infected?	Eggs per ovary* (% of total ovaries (n = 40) in each category)			
			None (no eggs)	Low (1–2 eggs)	Medium (3–9 eggs)	High (≥10 eggs)
<i>Sxl^{f4}/Sxl^{f4}</i>	Father	No	97%	3%	0	0
<i>Sxl^{f4}/Sxl^{f4}</i>	Mother	Yes	20%	18%	20%	42%

*Flies were dissected and scored as in Fig. 1.

†Reciprocal initial crosses were performed between an uninfected *w^{cm} Sxl^{f4} sn³/Binsinscy, y w Sxl⁺ sn³* B stock and a wild-type stock (Canton S) infected with the *y w* strain of *Wolbachia* to obtain homozygous mutant females at 25 °C for comparison four generations later, which differed only with respect to their infection status.

Table 2 Only a minority of *Wolbachia*-suppressed *Sxl^{f4}* females produce progeny

Genotype of infected sisters	Number of eggs laid per female in ten days* (% of total females in each category)				Females with offspring
	None	1–4	5–10	≥10	
<i>Sxl^{f4}/Sxl^{f4}</i>	30%	30%	15%	25%	10% (20)
<i>Sxl^{f4}/Sxl⁺</i>	15%	0%	5%	80%	85% (20)

Numbers in parentheses indicate number of females tested.

*Infected females from Table 1 were mated singly to groups of wild-type (Oregon R) males and allowed to lay at 25 °C for a total of ten days over three transfers to fresh food. Forty uninfected *Sxl^{f4}/Sxl^{f4}* females laid no eggs in ten days.

X-chromosome dosage compensation in *Drosophila melanogaster*¹². It is also required in the female germ line for oogenesis and meiotic recombination^{13,14}. Complete loss-of-function *Sxl* alleles are recessive, female-specific lethal; however, a small subset of partial loss-of-function point mutant alleles are disrupted specifically with respect to female germline functions of *Sxl* and hence are fully viable but sterile^{15,16}. Females mutant for such alleles fail to produce eggs owing to a germline-autonomous block in oogenesis that leads to overproliferation of undifferentiated germ cells¹³. The fact that the positive feedback loop for *Sxl* pre-messenger RNA splicing is engaged and mutant protein is produced in these ovarian tumours^{17,18} suggests that the overall level of *Sxl* protein in these mutant cells is normal.

A stock constructed for a suppressor screen of the female-sterile allele *Sxl^{f4}* was unusual in that homozygous mutant females were weakly fertile even before the screen began. These *Sxl^{f4}* females produced large numbers of eggs but few offspring. Three observations showed that the serendipitous suppressor carried in this stock was maternally inherited: (1) suppression could be passed through all mothers over several generations of outcrossing; (2) no chromosome of the original suppressed stock was required zygotically or maternally for suppression; and (3) males could not transmit suppression (data not shown).

Maternal inheritance of suppression led us to consider *Wolbachia* as a causative agent. We determined that the fertile *Sxl^{f4}* stock tested positive for *Wolbachia* by a polymerase chain reaction assay¹⁹, whereas the mutant stock from which the *Sxl^{f4}* chromosome had originally been obtained tested negative. Curing the fertile mutant stock of its bacterial infection by tetracycline treatment eliminated suppression (Fig. 1). Without tetracycline treatment, ovaries of homozygous mutant *Sxl^{f4}* females were full of eggs like the ovaries of their heterozygous *Sxl⁺* sisters. With tetracycline treatment, most *Sxl^{f4}* ovaries made no mature eggs, whereas the *Sxl⁺* ovaries of their sisters continued to produce normal numbers of eggs.

To establish that *Wolbachia* was indeed the bacterial agent causing suppression, we infected a female-sterile *Sxl^{f4}* line by crossing it to a wild-type (Canton S) line carrying a characterized strain of *Wolbachia*¹⁰. Because only the mother can transmit *Wolbachia*, reciprocal crosses could be used to create chromosomally identical *Sxl^{f4}/Sxl^{f4}* females differing only in their *Wolbachia* infection status. More than 60% of the mutant ovaries from twenty females infected this way produced more than two eggs, whereas only 3% of the mutant ovaries from twenty chromosomally identical but uninfected *Sxl^{f4}* females produced even one egg (Fig. 1b, c and Table 1).

Although most infected *Sxl^{f4}* females laid eggs, only a minority

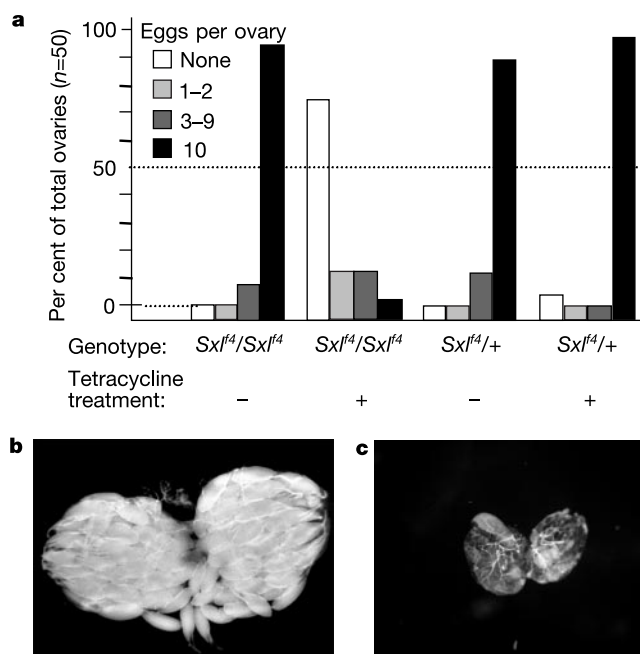


Figure 1 Egg production in *Sxl^{f4}/Sxl^{f4}* females is sensitive to tetracycline. **a**, Animals were raised at 25 °C on Carolina instant medium with or without 0.03 mg ml⁻¹ tetracycline. *Sxl^{f4}/Sxl^{f4}* and *Sxl^{f4}/+* sisters were dissected five days after eclosion. Eggs

reaching stage 13 (as indicated by presence of dorsal appendages) were counted. **b, c**, Ovaries from 4-day-old *Wolbachia*-infected (**b**) or uninfected (**c**) *Sxl^{f4}/Sxl^{f4}* females. Magnification 12 ×.

actually generated viable adult progeny over a ten-day period (Table 2). Among 610 eggs collected from a group mating of such females, 42% of the embryos reached at least the germband elongation stage, but only 7% hatched into larvae. This high level of embryonic lethality is probably caused at least in part by aneuploidy arising from meiotic chromosome segregation defects in *Sxl*^{f4} females that are not suppressed by *Wolbachia* infection. The X-chromosome non-disjunction rate ((2XXY + 2X0)/(XX + XY + 2XXY + 2X0)), where 0 indicates a missing Y chromosome) was 30% for *Sxl*^{f4}/*Sxl*^{f4} females (397 total offspring) compared with less than 0.3% for their *Sxl*^{f4}/*Sxl*^{f4}; *Dp* (*Sxl*⁺) sisters (1,587 total offspring). Such meiotic defects have been seen for a variety of heteroallelic mutant *Sxl* genotypes¹⁴ (T.W.C., unpublished data), and a similar phenotype has been reported for mutant *Sxl* females whose sterility was suppressed by uncharacterized chromosomal mutations²⁰. The fact that *Wolbachia* suppresses germline proliferation defects but not meiotic defects is evidence that the interaction with *Sxl* is functionally specific even within a single cell type.

The ability of *Wolbachia* to suppress female-sterile *Sxl* alleles depends on the specific molecular nature of those mutant alleles, rather than the severity of their germline defect. Such allele specificity is the strongest evidence for a direct involvement of the *Sxl* protein in suppression and argues against the possibility that *Wolbachia* suppresses by simply increasing the level of *Sxl* germline expression. Similar to *Sxl*^{f4}, the homozygous viable, female-sterile allele *Sxl*^{f5} is altered within a poly-proline tract that is present in all of the many *Sxl* protein isoforms¹⁷. In *Sxl*^{f5}, a leucine has replaced a proline only three residues away from the proline to serine change in *Sxl*^{f4}. We found that *Wolbachia* suppressed the oogenesis defect in *Sxl*^{f5} but less than in *Sxl*^{f4}, and it failed to suppress the germline phenotype of *Sxl*^{f5} when the allele also carried the phenotypically subtle, partial-loss-of-function mutation *Sxl*^{fHia} *in cis* (Table 3). *Sxl*^{fHia} is a spontaneous insertion of a *hobo* transposon in intron 2 at base pair 7,476 that arose unexpectedly in a *Sxl*^{f5} stock. Females homozygous for the double-mutant *Sxl*^{f5,fHia} are viable but sterile, like those homozygous for *Sxl*^{f5}; however, although *Sxl*^{f5} is fully viable *in trans* to a null allele, the double mutant is only poorly viable. As predicted, the non-suppressible double mutant allele had an effect on suppression in heteroallelic combination with *Sxl*^{f4} equivalent to that of the null allele *in trans* to *Sxl*^{f4} (Table 3), excluding the possibility that some genetic factor other than the

difference in *Sxl* alleles is responsible for the difference in suppression. Because the *Sxl*^{fHia} transposon insertion is in non-protein-coding DNA, its effects presumably stem from a reduction in *Sxl*^{f5} protein levels. Hence it seems that *Wolbachia* cannot suppress oogenesis defects if *Sxl*^{f5} germline protein levels fall below a certain threshold.

Wolbachia also suppressed the sterility of a molecularly different, homozygous viable, female-sterile allele, *Sxl*^{f18} (Table 3). However, *Sxl*^{f18} was suppressed more poorly than *Sxl*^{f4}, despite being less defective. The point lesion in *Sxl*^{f18} leaves the poly-proline tract unchanged, but substitutes aspartic acid for glycine in exon-8 carboxy-terminal isoforms and blocks alternative splicing that generates exon-10 C-terminal isoforms^{18,21}. Although none of the *Sxl* C-terminal isoforms seem to be germline-specific in their expression^{18,22}, the genetic behaviour of *Sxl*^{f18} indicates that exon-10 C-terminal isoforms are specifically required for oogenesis^{18,21}. The fact that egg production in uninfected *Sxl*^{f18} females was clearly better than that in uninfected *Sxl*^{f4} females shows that *Sxl*^{f18} is the less deleterious germline mutation; nevertheless, *Wolbachia*-infected *Sxl*^{f18} females made significantly fewer eggs than infected *Sxl*^{f4} females (Table 3). That the more functional mutant allele is the allele more weakly suppressed is also apparent from the behaviour of hemizygous mutant females—individuals who carry only a single copy of the mutant allele *in trans* to a completely non-functional (null) allele. Egg production in infected *Sxl*^{f18}/*Sxl*^{null} females was far below that for infected *Sxl*^{f4}/*Sxl*^{null} females (Table 3). Clearly, for both *Sxl*^{f18} and *Sxl*^{f4}, the dose of the mutant allele mattered, as egg production for infected homozygous mutant females was much greater than for infected hemizygous mutant females.

The fact that the mutant allele with the stronger oogenesis defect when uninfected would be the allele with phenotype closer to the wild type when infected argues against the possibility that suppression by *Wolbachia* arises from a general elevation of *Sxl* germline activity. The argument is even more compelling when one looks more carefully at the mutant allele dose effect. Egg production for infected *Sxl*^{f18}/*Sxl*^{null} females was far below that for even uninfected *Sxl*^{f18}/*Sxl*^{f18} females. If infection had been able to even double *Sxl*^{f18} expression, egg production for these two genotypes would have been the same. In contrast, although egg production for infected *Sxl*^{f4}/*Sxl*^{null} females was below that for infected *Sxl*^{f4}/*Sxl*^{f4} females, it was far above that for uninfected *Sxl*^{f4}/*Sxl*^{f4} females. Hence to account for the level of oogenesis in infected *Sxl*^{f4}/*Sxl*^{null} females, one would have to argue that *Wolbachia* increases *Sxl*^{f4} expression by much more than 100%, a proposal at odds with the results with *Sxl*^{f18}.

The conclusion that *Wolbachia* does not bypass or reduce the requirement for *Sxl* in the germline in a general way, nor increase overall germline *Sxl* expression, is also supported by its failure to suppress female-sterile mutations of other genes listed in Table 3, particularly *sans-fille*. Ovarian tumours resembling at least superficially those in *Sxl* mutants can be observed in females mutant for *sans-fille* (refs 23, 24), ovarian tumours (ref. 25) or *mei-P26* (ref. 26). The fact that none of these germline phenotypes were suppressed by *Wolbachia* infection is additional evidence for the specificity of the interaction with *Sxl*. Lack of suppression of *snf*¹⁶²¹ tumours is particularly significant, as these tumours are caused by an absence of germline *Sxl* activity, as shown by the fact that they can be suppressed by constitutively active mutant *Sxl* alleles²⁴, or even simply by an extra copy of *Sxl*⁺ (cited in ref. 27). If suppression of *Sxl* female-sterile mutations by *Wolbachia* were due to an increase in the expression of *Sxl*, rather than an effect on mutant *Sxl* protein function, *Wolbachia* should have suppressed *snf*¹⁶²¹, as do extra copies of *Sxl*⁺.

The effect of *Wolbachia* on *Sxl* functioning in *D. melanogaster* seems to be restricted to the germ line, as there is no evidence for the sex-specific lethality that would accompany inappropriate somatic

Table 3 *Wolbachia* is not a general suppressor of ovarian tumour mutants

Female genotype*	Females infected?†	Eggs per ovary (% of total ovaries (n = 40) in each category)			
		None (no eggs)	Low (1–2 eggs)	Medium (3–9 eggs)	High (≥10 eggs)
<i>Sxl</i> ^{f4} / <i>Sxl</i> ^{f4}	Yes	20%	18%	20%	42%
<i>Sxl</i> ^{f4} / <i>Sxl</i> ^{null}	Yes	30%	20%	35%	15%
<i>Sxl</i> ^{f4} / <i>Sxl</i> ^{f4}	No	97%	3%	0%	0%
<i>Sxl</i> ^{f5} / <i>Sxl</i> ^{f5}	Yes	43%	25%	25%	7%
<i>Sxl</i> ^{f5} / <i>Sxl</i> ^{null}	Yes	70%	5%	15%	10%
<i>Sxl</i> ^{f5} / <i>Sxl</i> ^{f5}	No	100%	0%	0%	0%
<i>Sxl</i> ^{f5,fHia} / <i>Sxl</i> ^{f5,fHia}	Yes	92%	8%	0%	0%
<i>Sxl</i> ^{f5,fHia} / <i>Sxl</i> ^{f4}	Yes	23%	28%	37%	12%
<i>Sxl</i> ^{f18} / <i>Sxl</i> ^{f18}	Yes	20%	28%	40%	12%
<i>Sxl</i> ^{f18} / <i>Sxl</i> ^{null}	Yes	95%	5%	0%	0%
<i>Sxl</i> ^{f18} / <i>Sxl</i> ^{f18}	No	77%	20%	3%	0%
<i>snf</i> ¹⁶²¹ / <i>snf</i> ¹⁶²¹	Yes	100%	0%	0%	0%
<i>mei-P26</i> ^{fs1} / <i>mei-P26</i> ^{fs1}	Yes	100%	0%	0%	0%
<i>otu</i> ¹¹ / <i>otu</i> ¹¹	Yes	77%	20%	3%	0%
<i>otu</i> ¹¹ / <i>otu</i> ¹¹	No	77%	23%	0%	0%
<i>Sxl</i> ^{f4} / <i>Sxl</i> ^{f4}	Yes	23%	25%	32%	20%

* *Sxl*^{null} is *Sxl*^{f1}. Data from Table 1 are repeated in rows one and three for convenience. Flies were raised at 25 °C. Infected *Sxl*^{f4}/*Sxl*^{null} females are cousins of the infected *Sxl*^{f4}/*Sxl*^{f4} females listed above them.

† The *Wolbachia* source for the eleven *Sxl* mutant genotypes in the upper part of the table was a wild-type (Canton S y w) infected stock, whereas that for the five lower genotypes was the *FM3*-balanced laboratory stock that originally introduced *Wolbachia* to *Sxl*^{f4} females inadvertently. As the *FM3* balancer chromosome carries recessive lethal mutations, it must normally be passed only through females, hence, it is possible that all stocks with *FM3* are infected with *Wolbachia*.

expression of *Sxl* (ref. 28). Moreover, using a very sensitive test²⁹, we determined that infection does not alter the effectiveness of the primary sex-determination signal (data not shown), perturbations of which can cause sex-specific lethality owing to inappropriate somatic expression of *Sxl*. Nevertheless, the possibility should be explored that *Wolbachia*-induced male killing reported for other *Drosophila* species⁶ may be caused by inappropriate activation of *Sxl*.

Although it may seem surprising that infection with a parasite would reverse the deleterious effect of a mutation in the host genome, particularly when the isolation of that mutation had nothing to do with infection, such surprise should be tempered by the fact that the interaction described here between host and parasite mimics a naturally occurring situation mentioned above that was reported recently for the parasitic wasp *Asobara tabida*⁹. Moreover, in light of the fact that *Wolbachia* is a parasite that is known to manipulate host reproductive and sex-determination systems, it does not seem unreasonable that the host gene with which it interacts in *Drosophila* is the master regulator of sex-determination and a gene essential for oogenesis. The fact that the interacting gene in this case has been studied so extensively and belongs to a model experimental organism can be exploited to yield further insights into the mechanism by which this parasite takes advantage of its various arthropod hosts. □

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- Werren, J. H. Biology of *Wolbachia*. *Annu. Rev. Entomol.* **42**, 587–609 (1997).
- Knight, J. Meet the Herod bug. *Nature* **412**, 12–14 (2001).
- Werren, J. H. & O'Neill, S. L. in *Influent Passengers: Inherited Microorganisms and Arthropod Reproduction* (eds O'Neill, S. L., Hoffman, A. A. & Werren, J. H.) 1–41 (Oxford Univ. Press, Oxford, 1997).
- Huigens, M. E. *et al.* Infectious parthenogenesis. *Nature* **405**, 178–179 (2000).
- Bouchon, D., Rigaud, T. & Juchault, P. Evidence for widespread *Wolbachia* infection in isopod crustaceans: molecular identification and host feminization. *Proc. R. Soc. Lond. B* **265**, 1081–1090 (1998).
- Hurst, G. D. D., Johnson, A. P., Schulenburg, J. H. G. & Fuyama, Y. Male-killing *Wolbachia* in *Drosophila*: a temperature-sensitive trait with a threshold bacterial density. *Genetics* **156**, 699–709 (2000).
- Boyle, L., O'Neill, S. L., Robertson, H. M. & Karr, T. L. Interspecific and intraspecific horizontal transfer of *Wolbachia* in *Drosophila*. *Science* **260**, 1796–1799 (1993).
- Bordenstein, S. R., O'Hara, F. P. & Werren, J. H. *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in *Nasonia*. *Nature* **409**, 707–710 (2001).
- Dedeine, F. *et al.* Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *Proc. Natl Acad. Sci. USA* **98**, 6247–6252 (2001).
- Bourtzis, K., Nirgianaki, A., Markakis, G. & Savakis, C. *Wolbachia* infection and cytoplasmic incompatibility in *Drosophila* species. *Genetics* **144**, 1063–1073 (1996).
- Min, K. T. & Benzer, S. *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. *Proc. Natl Acad. Sci. USA* **94**, 10792–10796 (1997).
- Cline, T. W. & Meyer, B. J. Vive la différence: males vs females in flies vs worms. *Annu. Rev. Genet.* **30**, 637–702 (1996).
- Schupbach, T. Normal female germ cell differentiation requires the female X-chromosome to autosome ratio and expression of *Sex-lethal* in *Drosophila melanogaster*. *Genetics* **109**, 529–548 (1985).
- Cook, K. R. *Regulation of Recombination and Oogenesis by the ovarian tumor, Sex-lethal, and ovo Genes of Drosophila melanogaster*. Thesis no. 381, Univ. Iowa (1993).
- Salz, H. K., Cline, T. W. & Schedl, P. Functional changes associated with structural alterations induced by mobilization of a P element inserted in the *Sex-lethal* gene of *Drosophila*. *Genetics* **117**, 221–231 (1987).
- Perrimon, N., Mohler, D., Engstrom, L. & Mahowald, A. P. X-linked female-sterile loci in *Drosophila melanogaster*. *Genetics* **113**, 695–712 (1986).
- Bopp, D., Horabin, J. I., Lersch, R. A., Cline, T. W. & Schedl, P. Expression of the *Sex-lethal* gene is controlled at multiple levels during *Drosophila* oogenesis. *Development* **118**, 797–812 (1993).
- Dines, J. L. *New Aspects of Functional Complexity for the Master Regulator of Drosophila melanogaster Sex Determination* Thesis no. 319, Univ. California, Berkeley (2001).
- O'Neill, S. L., Giordano, R., Colbert, A. M. E., Karr, T. L. & Robertson, H. M. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. *Proc. Natl Acad. Sci. USA* **89**, 2699–2702 (1999).
- Bopp, D., Schutt, C., Puro, J., Huang, H. & Nothiger, R. Recombination and disjunction in female germ cells of *Drosophila* depend on the germline activity of the gene *Sex-lethal*. *Development* **126**, 5785–5794 (1999).
- Dines, J., Lersch, B., Lu, B., Bell, M. & Cline, T. W. Functional specialization of SEX-LETHAL protein isoforms. *Annu. Drosophila Res. Conf. Program Abs. Vol. 39*, a245 (1998).
- Salz, H. K. *et al.* The *Drosophila* female-specific sex-determination gene, *Sex-lethal*, has stage-, tissue-, and sex-specific RNAs suggesting multiple modes of regulation. *Genes Dev.* **3**, 708–719 (1989).
- Oliver, B., Perrimon, N. & Mahowald, A. P. Genetic evidence that the *sans fille* locus is involved in *Drosophila* sex determination. *Genetics* **120**, 159–172 (1988).
- Steinmann-Zwicky, M. Sex determination in *Drosophila*: the X-chromosomal gene *liz* is required for *Sxl* activity. *EMBO J.* **7**, 3889–3898 (1988).
- Pauli, D., Oliver, B. & Mahowald, A. P. The role of the ovarian tumor locus in *Drosophila melanogaster* germline sex determination. *Development* **119**, 123–134 (1993).

- Page, S. L., McKim, K. S., Deneen, B., Van Hook, T. L. & Hawley, S. R. Genetic studies of *mei-P26* reveal a link between the processes that control germ cell proliferation in both sexes and those that control meiotic exchange in *Drosophila*. *Genetics* **155**, 1757–1772 (2000).
- Hager, J. H. & Cline, T. W. Induction of female *Sex-lethal* RNA splicing in male germ cells: implications for *Drosophila* germline sex determination. *Development* **124**, 5033–5048 (1997).
- Cline, T. W. A male-specific lethal mutation in *Drosophila melanogaster* that transforms sex. *Dev. Biol.* **72**, 266–275 (1979).
- Cline, T. W. Evidence that *sisterless-a* and *sisterless-b* are two of several discrete 'numerator elements' of the X/A sex determination signal in *Drosophila* that switch *Sxl* between two alternative stable expression states. *Genetics* **119**, 829–862 (1988).

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Sequence and analysis of chromosome 2 of *Dictyostelium discoideum*

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The genome of the lower eukaryote *Dictyostelium discoideum* comprises six chromosomes. Here we report the sequence of the largest, chromosome 2, which at 8 megabases (Mb) represents about 25% of the genome. Despite an A + T content of nearly 80%, the chromosome codes for 2,799 predicted protein coding genes and 73 transfer RNA genes. This gene density, about 1 gene per 2.6 kilobases (kb), is surpassed only by *Saccharomyces cerevisiae* (one per 2 kb) and is similar to that of *Schizosaccharomyces pombe* (one per 2.5 kb)^{1,2}. If we assume that the other chromosomes have a similar gene density, we can expect around 11,000 genes in the *D. discoideum* genome. A significant number of the genes show higher similarities to genes of vertebrates than to those of other fully sequenced eukaryotes^{1–6}. This analysis strengthens the view that the evolutionary position of *D. discoideum* is located before the branching of metazoa and fungi but after the divergence of the plant kingdom⁷, placing it close to the base of metazoan evolution.

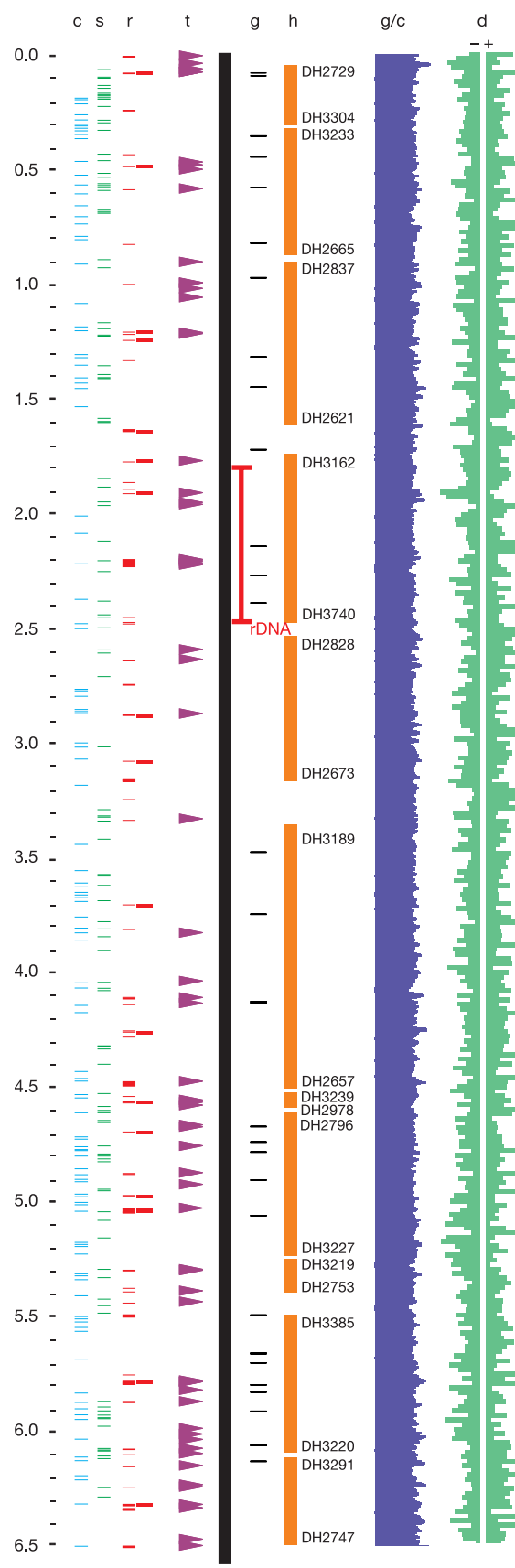


Figure 1 Feature distribution on chromosome 2. Only the linked portion (6.5 Mb) is shown (solid black line). Clone gaps (c), sequence gaps (s), repeat elements (r; heavier bars to their right indicate unresolvable clusters), tRNA genes (t) and genes (g) used to seed assembly are shown. HAPPY linkage groups (h) were used to guide assembly; only the endmost markers in each group are named. G+C content (g/c), strand-specific coding

sequence density (d), the ribosomal DNA copy, and the duplicated region above it (represented here as a single copy) are shown. The centromere and telomere are respectively above and below the portion shown. An expanded version is at <http://genome.imb-jena.de/dictyostelium/chr2/Chr2map.html>.

The natural habitat of *D. discoideum* is deciduous forest soil where the amoeboid cells feed on bacteria by phagocytosis and multiply by equal mitotic division. Exhaustion of the food source triggers a developmental programme, in which more than 100,000 cells aggregate by chemotaxis to form a multicellular structure. Morphogenesis and cell differentiation then culminate in the production of spores, enabling the organism to survive unfavourable conditions⁸. *D. discoideum* therefore lies at the borderline between free-living cells and multicellular organisms, making it ideal for the study of cellular differentiation and integration. Its haploid genome, ease of culture and genetic manipulability make it amenable to biochemical, genetic, and cell-biological approaches⁹. This allows the dissection of the molecular basis of the most fundamental cellular processes: differentiation, signal transduction, phagocytosis, cytokinesis, cell motility and chemotaxis^{10–12}.

To provide the basis for genome-wide investigations an international effort was initiated¹³ to sequence the ~34-Mb genome of *D. discoideum*, strain AX4. Besides six chromosomes ranging from 4 to 8 Mb (refs 14, 15), the nucleus harbours approximately 100 copies of a ~90-kb palindromic chromosome containing the ribosomal RNA genes. The high A+T content (78%, exceeded only by *Plasmodium falciparum* at 80%; refs 16, 17) coupled with a high density of repetitive elements, posed severe challenges for genome sequencing. To reduce the complexity of the assembly task, the genome was analysed chromosome by chromosome, using a whole chromosome shotgun (WCS) approach. The chromosomal libraries were only ~50% pure and contained clones derived from other chromosomes, so we developed an iterative and integrated assembly strategy. This allowed us to identify contiguous DNA sequences (contigs) originating from chromosome 2 and to bridge difficult sequences. Briefly (see Methods), nonrepetitive reads from the chromosome 2-enriched libraries were binned with those from the other WCS projects, and sequences of known chromosome 2 genes were used as 'seeds' around which to build contigs. These were extended using sequence data and supplemented using read-pair

information and BLAST (<http://blast.wustl.edu/>) analysis. To confirm the chromosomal assignment of these contigs we used the relative frequencies of the constituent sequences in the chromosomally enriched libraries of the various WCS projects.

The high A+T content, the existence of many repetitive elements and the fact that clones larger than about 5 kb were unstable in *Escherichia coli*^{18,19}, precluding the use of large-insert bacterial clones as second-source templates, led to three types of gaps. The first type could not be spanned by plasmid clones ('clone gaps'), presumably owing to the instability of some of the intergenic regions, which have A+T contents of up to 98%. The second type arose from clusters of repetitive elements, which could not be unambiguously resolved ('repeat gaps'). The third type ('sequence gaps') were spanned by clones which, owing to their content of long homopolymer runs (even more abundant and longer than in *P. falciparum*) or lack of targets for custom primers, were recalcitrant to repeated attempts at sequencing. Contigs divided by sequence gaps were linked by read-pair information to produce larger 'scaffolds' with a total size of 7.5 Mb. The majority of these scaffolds were then connected, oriented and their internal structure validated by using mapped genes, circular yeast artificial chromosomes (cYACs) and HAPPY map²⁰ data. This yielded a 'linked portion' spanning 6.5 Mb of the chromosome (Fig. 1; Table 1; and Supplementary Information; see also <http://genome.imb-jena.de/dictyostelium/chr2/Chr2map.html>). Although many of the sequence and

Table 1 Features of chromosome 2

Feature	Value
Calculated total length (Mb)*	8.0–8.1
Total length of sequence contigs (Mb)*	7.52
Cumulated length of 71 small orphan unlinked contigs (Mb)	0.4
Number of loci containing complex repetitive elements**	58
Resolved loci	40
Unresolved loci	18
Number of tRNAs	73
Genes†	
Predicted number	2,799
Density	1 gene/2.6 kb
Average length (bases)	1,626
Number of genes with ESTs	1,120 (40%)
AT content (%)	
Exons	72
Introns	87
Intergenic	86
Whole chromosome	77.8
Exons (coding)	
Number	6,398
Average exon number/gene	2.29
Average size (bases)	711
Introns	
Number	3,587
Average size (bases)	177
Intergenic regions	
Average size (bases)	786
Intronless genes (%)	893 (32)

*Excluding duplication of 0.7 Mb.

**In 6.5-Mb linked portion of chromosome 2.

†Excluding genes coded for in repeat loci.

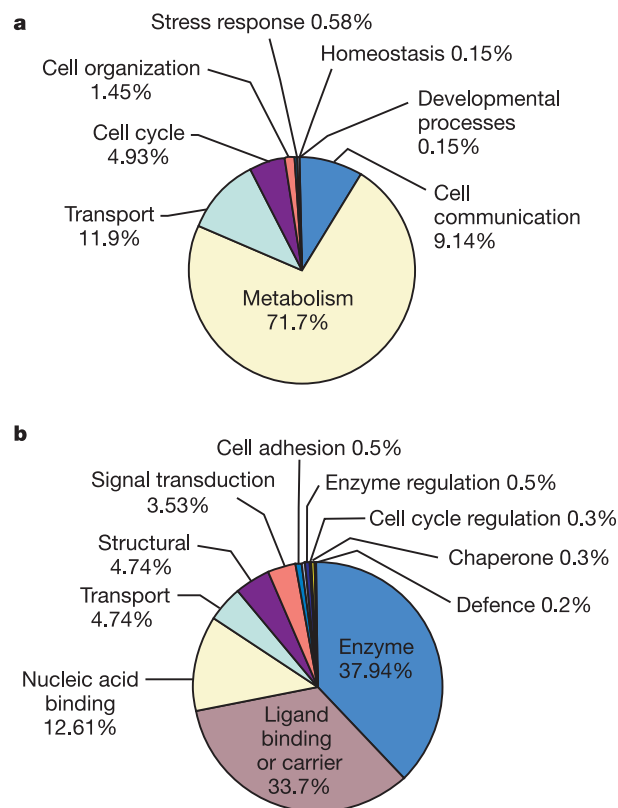


Figure 2 Functional classification of *D. discoideum* chromosome 2-coded proteins. We used the GO terminology (<http://whitefly.lbl.gov/annot/go/database/index.html>) for the automated classification of proteins in process (a) and function (b) groups according to their InterPro domains. The process groups contain 689 proteins, the function groups 991 proteins. Proteins with InterPro domains but no GO assignment (424) or proteins without Interpro domains (1,319) were not characterized. Currently no *D. discoideum*-specific GO terms are defined, thus leaving some of the functionally characterized *D. discoideum*-specific genes unclassified.

clone gaps have been closed, those that remain (95 sequence gaps and 89 clone gaps, totalling an estimated 150 kb) appear intractable. The most resistant gaps have been those containing the most A+T-rich DNA, and are hence least likely to contain sequences of biological relevance.

D. discoideum chromosomes have been reported to be acro- or telocentric with the centromere embedded in a large cluster of long terminal repeat retrotransposons (DIRS-1) composed of more than 40 elements^{15,19}. The fine structure of this cluster, which lies outside the linked portion of the chromosome shown in Fig. 1, could not be resolved because of the low polymorphism rates of the complex repetitive elements¹⁹. It spans up to 0.5 Mb and also contains copies from other transposon families and small repetitive elements. Overall, the number of repetitive elements in *D. discoideum* genomic DNA is high compared to *S. cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster*. Chromosome 2 harbours all previously described *D. discoideum* complex repetitive elements, mainly organized in clusters of intact and truncated elements¹⁹. There are 58 such loci (each consisting of one or more such elements) on the linked portion of chromosome 2. The fine structure of 18 of these could not be resolved and remain as 'repeat gaps' (Fig. 1; Table 1). Altogether, we estimate that complex repetitive elements represent 10.2% (approximately 0.8 Mb) of chromosome 2, corresponding well with the estimate of 9.6% for the entire genome¹⁹. On the basis of the combined sizes of the sequence scaffolds, the clone and sequence gaps, and the unresolved repeat regions (including the pericentromeric region), we calculate the size of the chromosome to be 8.1 Mb.

A duplication of approximately 700 kb is thought to have occurred after the separation of the laboratory strains AX2 and AX4 (ref. 15). We detected an inverse tandem repeat of similar size between the HAPPY Map markers DH3162 and DH3740, bordered at the telomeric end by an almost complete copy of the extrachromosomal rDNA palindrome (Fig. 1). This might represent a chromosomal master copy for the generation of the

extrachromosomal rDNA palindrome after sexual recombination, as in *Tetrahymena thermophila*²¹. The second copy of the duplication was excluded from calculations of chromosome length and gene number.

We find that most features of the chromosome (G+C content, coding sequence density (CDS) and complex repetitive elements) are evenly distributed over the 6.5-Mb linked portion, although the distribution of the transfer RNA genes shows a slight bias towards the telomere (Fig. 1; <http://genome.imb-jena.de/dictyostelium/chr2/Chr2map.html>). We used gene prediction programs and database searches to determine and annotate the 2,799 putative genes of chromosome 2. A further 124 putative genes coded for by complex repetitive elements were excluded from further analyses. *D. discoideum* genes in general have few and small introns, with an average of 1.2 introns per gene. Intron length and distribution is comparable to that of *P. falciparum* and other lower eukaryotes. The mean A+T content in exons is 72%, whereas it is 87% in introns, and 86% in intergenic regions (Table 1). This extreme compositional bias may help to delineate the introns during splicing, as has been suggested in *Arabidopsis thaliana*. In support of this hypothesis, *D. discoideum* introns do contain the canonical GT-AG dinucleotides but, unusually among fully sequenced eukaryotes, all information is confined to the intron side of the splice site²².

Turning to the gene content, expressed sequence tags (ESTs) exist for 40% (1,120) of the predicted genes (Table 1)²³. BLAST searches against the protein sets of completely sequenced eukaryotic genomes as well as against SwissProt and TrEMBL databases showed that 45% (1,260) of the putative *D. discoideum* genes had a match ($P < 10^{-15}$), leaving the proportion of unique genes (55%) comparable to that observed for other eukaryotes. About 53% (1,480) of the putative genes contained domains defined in the InterPro database (<http://www.ebi.ac.uk/interpro>)²⁴; again, this proportion is comparable to other eukaryotes⁶. In total, EST, protein, and/or InterPro matches provide support for 1,960 of the 2,799 predicted

Table 2 Most frequent InterPro domains

Domain	Description	DD	SC	AT	CE	DM	HS
IPR001687	ATP/GTP-binding site motif A (P-loop)*	6.07	0.57	0.61	0.32	0.46	0.33
IPR000694	Proline-rich region	3.72	NA	NA	NA	NA	NA
IPR000561	EGF-like domain*	2.18	0.02	0.16	0.68	0.62	1.28
IPR000719	Eukaryotic protein kinase	1.93	1.91	4.07	2.34	1.79	2.64
IPR002290	Serine/threonine protein kinase	1.89	1.83	3.34	1.33	1.22	1.83
IPR001245	Tyrosine protein kinase	1.71	0.05	1.84	0.84	0.65	1.22
IPR001680	G-protein beta WD-40 repeats	1.11	1.63	1.02	0.80	1.31	1.34
IPR003593	AAA ATPase superfamily*	1.11	0.95	0.90	0.40	0.56	0.46
IPR000051	SAM (and some other nucleotide) binding motif	0.89	0.33	0.40	0.25	0.28	0.20
IPR001849	Pleckstrin homology (PH) domain	0.89	0.47	0.12	0.41	0.54	1.24
IPR002048	EF-hand*	0.86	0.26	0.85	0.65	0.93	1.15
IPR001841	RING finger	0.82	0.65	1.82	0.81	0.85	1.20
IPR002085	Zinc-containing alcohol dehydrogenase superfamily	0.82	0.34	0.15	0.06	0.07	0.08
IPR000794	Beta-ketoacyl synthase*	0.79	0.03	0.02	0.02	0.03	0.01
IPR003579	RAS small GTPases, Rab subfamily	0.79	0.15	0.23	0.15	0.21	0.22
IPR001611	Leucine-rich repeat	0.75	0.13	1.93	0.33	0.83	0.74
IPR003577	RAS small GTPases, Ras subfamily	0.75	0.05	0.00	0.06	0.07	0.10
IPR003880	Phosphopantetheine attachment site	0.75	0.10	0.21	0.15	0.28	0.08
IPR000504	RNA-binding region RNP-1 (RNA recognition motif)	0.71	0.93	0.96	0.69	1.13	1.25
IPR000873	AMP-dependent synthetase and ligase	0.71	0.18	0.17	0.17	0.25	0.16
IPR003578	RAS small GTPases, Rho subfamily	0.71	0.10	0.04	0.05	0.04	0.10
IPR000345	Cytochrome c family haem-binding site	0.68	0.10	0.58	0.31	0.31	0.37
IPR001227	Acyl transferase domain*	0.68	0.02	0.00	0.02	0.03	0.01
IPR001806	Ras GTPase superfamily	0.68	0.36	0.38	0.33	0.51	0.60
IPR000477	RNA-directed DNA polymerase (Reverse transcriptase)	0.64	0.08	0.50	0.46	0.09	0.14
IPR001064	Zinc-finger GCS-type	0.64	0.10	0.07	0.04	0.07	0.14
IPR002110	Ankyrin-repeat*	0.64	0.29	0.44	0.53	0.62	0.91
IPR001601	Generic methyl-transferase	0.61	0.10	0.22	0.06	0.07	0.06
IPR001410	DEAD/DEAH box helicase	0.57	1.19	0.53	0.44	0.54	0.52
IPR002106	Aminoacyl-transfer RNA synthetases class-II	0.57	0.36	0.35	0.59	0.41	0.20

Occurrence of the thirty most frequent InterPro domains on *D. discoideum* chromosome 2 (DD; including repetitive elements) and fully sequenced eukaryotes. The percentage of genes in each organism that contain the respective domain type is given. SC, *S. cerevisiae*; AT, *A. thaliana*; CE, *C. elegans*; DM, *D. melanogaster*; HS, *H. sapiens*. The data for SC, CE, DM, HS and AT were taken from <http://www.ebi.ac.uk/interpro/>. NA, not analysed.

*These entries are discussed in the text.

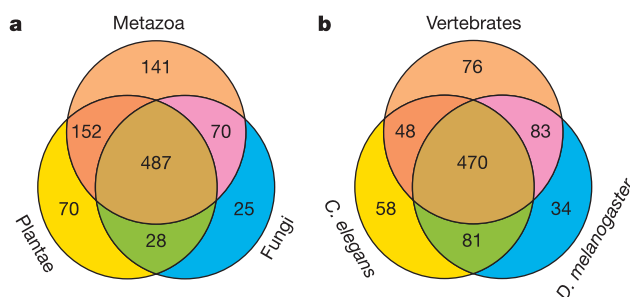


Figure 3 Phylum-specific distribution of proteins. **a**, For comparison with the chromosome 2 translated genes, plants are represented by the full protein set of *A. thaliana* and fungi by *S. cerevisiae* plus *S. pombe*. Metazoans are represented by *D. melanogaster*, *C. elegans*, the (as yet incomplete) *Homo sapiens* protein set, plus annotated nonhuman vertebrate proteins from the SwissProt database. **b**, Distribution of *D. discoideum* genes with $P < 10^{-15}$ between fully sequenced metazoan species. The vertebrate gene set is as in **a**.

genes. Applying the gene ontology (GO) terminology for the automated classification of proteins (<http://whitefly.lbl.gov/annot/go/database/index.html>) we could attribute functions and/or processes to 1,026 (37%) of the predicted protein products (Fig. 2). Slightly more of these proteins could be classified to functions (991) than to process (689) categories, and 654 out of the 1,026 classified proteins are present in both categories. Forty-seven per cent of the putative proteins remain unclassified and a further 15% of all proteins could not be categorized because their corresponding InterPro domains are not yet assigned to GO terms (Fig. 2).

Because *D. discoideum* undergoes differentiation and development we might expect a significant number of genes associated with multicellular life. In fact, a remarkably high proportion of GO-classified proteins are grouped into the cell communication category (9.14%) and involved in signal transduction or cell adhesion, or comprise cytoskeletal proteins containing signalling domains. As expected, analysis of InterPro matches reveals that domains required for cell motility, signalling, surface attachment and cytoskeletal functions are considerably more abundant than in yeast. When we compare the most frequent InterPro domains of chromosome 2 genes to those of other species (Table 2), the ATP/GTP-binding site motif A (P-loop) is strongly over-represented (6.07% of the predicted genes on the *D. discoideum* chromosome 2 carry this motif, versus 0.33% in human), whereas the epidermal growth factor (EGF)-like domain is over-represented only slightly compared to human (2.18% versus 1.28%), but strongly in comparison to yeast (0.02%). The AAA ATPase superfamily domain is found in comparable proportions in *D. discoideum*, *S. cerevisiae* and

A. thaliana, but is less abundant in *C. elegans*, *D. melanogaster* and human, whereas the proportions of the Ca^{2+} -binding EF-hand domain and the ankyrin repeat are roughly comparable in all organisms with the exception of yeast, where they are less abundant. We have also identified many beta-ketoacyl synthase and acyl transferase domains, which are hardly present in the other organisms considered here. In *D. discoideum* many of these domains are part of polyketide synthases, which are exceptionally large, multi-functional proteins, primarily present in actinomycetes, bacilli and filamentous fungi. The compounds built via the polyketide synthase pathways might enable *D. discoideum* to defend itself against its natural competitors.

Many *D. discoideum* genes—particularly those involved in signalling and cell movement—are known to be present as multiple copies or as members of large gene families. This is supported by our analysis of chromosome 2, which contains 130 genes present as two or more copies ($P < 10^{-30}$ and sequence similarity over the complete length), amounting to 337 (12%) of the predicted genes. Because paralogues on the other chromosomes have not been taken into account, the number of singletons will further decrease when all chromosomes have been analysed. We have found ten genes for members of the Ras-related small GTP-binding protein family and nine genes sharing the RasGEF domain. Furthermore, we identified another G protein with homology to Gα2, a component of the cyclic AMP signalling system, and also residing on chromosome 2. We have also found two more members of the G-protein coupled receptor family. Surprisingly, these proteins have highest homology to GABA (γ-aminobutyric acid) receptors, which have not yet been found outside the metazoan branch. Genes coding for components of the cytoskeleton are frequently present in multiple copies. Of the ~27 actin genes (including pseudogenes) in the *D. discoideum* genome¹⁹, thirteen are present on chromosome 2 and ten of these translate into identical protein sequences. Chromosome 2 harbours several genes coding for motor proteins, among which are six genes for different unconventional myosins. Although the cytoskeleton has been intensively studied, we have found putative new paralogues for profilin I/II, fimbrin, cofilin 1/2 and the unconventional myosin gene family. The discovery of additional putative paralogues of cytoskeletal proteins supports the concept of functional redundancy in the cytoskeletal system¹². The ABC (ATP-binding cassette) transporter family is probably one of the largest in the genome. There are thirteen such genes on chromosome 2, including several members of the ABC A subfamily whose occurrence has been restricted to multicellular eukaryotes. ABC transporters use the energy of ATP hydrolysis to translocate specific substrates across cellular membranes. Mutations in many of the human genes coding for ABC transporters are associated with disease such as cystic fibrosis, Stargardt's disease or hyperinsulinism. We have found genes on chromosome 2 with high similarities to the

Table 3 *D. discoideum* chromosome 2 genes with similarity to human disease genes

Disease (gene symbol)	OMIM number	Accession number	<i>D. discoideum</i> gene	BLASTP value ($< 1.0 \times 10^{-50}$)
Renal tubular acidosis (ATP6B1)	192132	AAD11943	dd_01070	4.0e-246 (0)
Immunodeficiency (DNA Ligase 1)	126391	NP_000225	dd_02463	1.9e-245 (0)
Hereditary nonpolyposis colorectal cancer, type 1 (HNPCC) (MSH2)	120435	AAA18643	dd_00995	2.4e-237 (1)
*Hyperinsulinism (ABCC8)	600509	Q09428	dd_00006	3.0e-220 (1)
G6PD deficiency (G6PD)	305900	NP_000393	dd_01534	5.1e-190 (0)
*Stargardt's (ABCA4)	601691	AAC51144	dd_02412	6.5e-189 (3)
Deafness, hereditary (MYO15)	602666	AAF05903	dd_02568	1.2e-182 (5)
Familial cardiac myopathy (MYH7)	160760	P12883	dd_02401	4.2e-177 (5)
Chediak-Higashi (CHS1)	214500	NP_000072	dd_02608	3.3e-151 (1)
Cancer (AKT2)	164731	AAA58364	dd_02928	1.5e-94 (2)
HNPCC (MSH3)	600887	AAB06045	dd_01030	8.9e-78 (1)

From a list of 287 confirmed human disease protein sequences³⁰ those are shown that match a *D. discoideum* chromosome 2 protein with a BLASTP probability of less than 1.0×10^{-50} , indicating a strong similarity. Only the best hit is listed and the total number of additional strong hits ($P < 1.0 \times 10^{-50}$) is given in parentheses after the probability score. OMIM, Online Mendelian Inheritance in Man (<http://www.ncbi.nlm.nih.gov/omim/>).

*Homologous proteins discussed in the text.

latter two genes and to several other human disease-related genes (Table 3).

What can the genome of *D. discoideum* tell us about the common genomic repertoire of eukaryotic life? To address this question, we compared the protein products of the 2,799 genes of chromosome 2 to the complete protein sets of fully sequenced eukaryotes ($P < 10^{-15}$) and found that 973 proteins (35%) have matches. Of these only 487 share similarities across plants (*A. thaliana*), fungi (*S. cerevisiae* and *S. pombe*) and metazoa (*C. elegans*, *D. melanogaster* and available vertebrate sequences). A surprisingly high number (141) have matches with metazoa but not plants or fungi (Fig. 3a). Subdividing metazoa into fly, worm and vertebrates shows that even amongst this closely related group not all genes have comparable similarities in each species (Fig. 3b). This may reflect gene losses during evolution, or evolutionary rate variations for identical genes in different organisms, but could also reflect a gain of function for specific gene groups in each organism. If chromosome 2 is taken as a representative quarter of the *D. discoideum* genome, then less than 2,400 different genes are shared between *D. discoideum*, *S. pombe*, *S. cerevisiae*, *C. elegans*, *A. thaliana*, *D. melanogaster* and man. This number might well represent the 'minimal gene set' of a free-living eukaryote. From random mutagenesis studies it was previously estimated that the essential genes of yeast comprise only about 30% of all its genes²⁵. Our estimate of the number of genes shared by all eukaryotes is close to this number.

Our analyses are all predicated on the assumption that chromosome 2, representing 25% of the genome, is typical of the remainder. This seems a reasonable assumption and other evidence on the distribution of mapped genes¹⁵ does not suggest that chromosome 2 is particularly atypical. Our findings can also clarify the evolutionary position of *D. discoideum*. Its genome exhibits greater similarities to metazoa than to plants or fungi (Fig. 3). This supports the finding of a recent phylogenetic analysis of conserved protein sequences which placed the Myxomycota (to which *D. discoideum* belongs) at a position before the branching of the metazoa and fungi but after the divergence of the plant kingdom⁷. *D. discoideum* does not appear to have suffered the extensive gene loss observed in *S. cerevisiae* and therefore its gene content may better represent a basic eukaryotic genome. This conservation of the complete gene set makes *D. discoideum* well suited for functional studies of genes not represented in yeast. Its surprisingly high gene number may in part reflect the higher order of complexity associated with multicellular life. □

Methods

Further information on sequence data and analysis results can be accessed via <http://genome.imb-jena.de/dictyostelium/> and <http://www.uni-koeln.de/dictyostelium/>.

Sequencing and assembly

Library construction and sequencing was done as described previously¹⁹. 160,000 chromosome 2 library-derived reads were pooled with the nonrepetitive reads from other *D. discoideum* whole chromosome shotgun projects to give a total of 500,000 reads. The subset of reads matching genes mapped to chromosome 2 (ref. 15) were assembled to build seed contigs, and further contigs were assembled around complementary reads from the clones in these seeds (for details see <http://genome.imb-jena.de/dictyostelium/chr2/seeds.html>). The previously published¹⁵ map order of the 'seed' genes was largely confirmed. Considering the combined data of the HAPPY map and sequence assembly, it is likely that the discrepancies arise from errors in the earlier YAC contigs, which have been shown to suffer from a proportion of misplaced clones²⁶. The assembly database was then enlarged by the incorporation of reads with a higher than average frequency of occurrence in the chromosome 2 library reads (these are more likely to originate from chromosome 2 than from other chromosomes, owing to our preferential use of the chromosome 2 specific clone libraries). The contigs were extended by the incorporation of further reads which were found by BLAST analysis of the contig ends. This assembly method yielded about 1,100 contigs larger than 2 kb. Chromosomal assignment of each contig was checked on the basis of its content of sequences derived from each of the different chromosome-enriched libraries (K.S., unpublished software). In this way, contaminant sequences were filtered out; conversely, reads derived from the other whole chromosome shotgun projects but assigned to chromosome 2 were incorporated into our assembly. To ensure that we had not missed portions of the chromosome by this strategy we assembled all chromosome 2 library-derived reads and checked the resulting contigs for chromosome specificity. The resulting additional contigs were added to the chromosome 2-specific assembly. All

contigs were manually inspected to ensure data accuracy. Clones spanning sequencing gaps between neighbouring contigs defined scaffolds. Directed closure of these gaps was done using custom primers to walk on existing clones. Additional gaps were closed by using the transposon insertion technique and polymerase chain reaction (PCR) approaches.

Mapping

As part of the ongoing genome-wide *D. discoideum* HAPPY mapping project, a short range (~100 kb), high-resolution (mean, 15 kb) HAPPY mapping panel was prepared from AX4 genomic DNA, pre-amplified by PEP (primer extension pre-amplification) and diluted before use as a template for marker typing. Hemi-nested primers were designed for 824 markers selected from the sequencing projects. Markers were typed onto the HAPPY mapping panel and sorted into linkage groups as previously described²⁶. Maps for each linkage group were generated and validated by inspection to reduce the risk of incorporating spurious intermarker linkages. The results obtained so far define the order of 365 chromosome 2 markers in 12 large linkage groups (for details see <http://genome.imb-jena.de/dictyostelium/chr2/linkage.html>). Further positional information was obtained from PCR screening of a cYAC library with insert sizes of 80–100 kb covering the genome approximately sevenfold. Sequence contigs or HAPPY linkage groups were assumed to be linked if primer pairs derived from them hit the same cYAC clone(s). By integrating the HAPPY, cYAC and sequence scaffold data, a region spanning 6.5 Mb was robustly assembled. Only four sequence scaffolds, totalling 0.6 Mb, could not be placed onto the map. Of these, two are too large to fit in the gaps of the linked 6.5-Mb portion of the chromosome, and are presumably located at the ends. The assembly produced 71 unlinked orphan contigs amounting to 0.41 Mb, consisting mainly of fragments of complex repetitive elements.

Sequence analysis

G+C content was calculated using a sliding window of 10,000 bases and a step size of 1,000 bases. Strand-specific CDS density was measured as percentage of coding triplets in a stepped window of 5,000 bases. A database containing the complex repetitive elements of *D. discoideum* was used with RepeatMasker to scan the sequence for repeats¹⁹. tRNAs were detected using tRNAscan-SE²⁷. To define the genes on chromosome 2, the gene prediction program GeneID²⁸ was trained with 140 known *D. discoideum* genes and its parameters adjusted to be able to define proper gene borders and intron positions. The lower limit for the gene length was 120 bases of coding sequence. EST matches were defined by BLAST with >98% identity, and word length of 32. The protein products of predicted genes were compared to the databases of completed genomes: The Arabidopsis Information Resource (<http://www.arabidopsis.org/home.html>), Wormpep (http://www.sanger.ac.uk/Projects/C_elegans/wormpep/), ftp://ftp.ebi.ac.uk/pub/databases/edgp/sequence_sets/, Saccharomyces Genome Database (<http://genome-www.stanford.edu/Saccharomyces/>), The Schizosaccharomyces pombe Genome Sequencing Project (http://www.sanger.ac.uk/Projects/S_pombe/), Ensembl Genome Browser (<http://www.ensembl.org>) as well as against SWISS-PROT entries and TrEMBL. They were also checked for the presence of InterPro domains using the InterPro database (<http://www.ebi.ac.uk/interpro>). Functional classification was done automatically using the GO classification system (<http://www.geneontology.org/>)²⁹.

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- Goffeau, A. et al. Life with 6000 genes. *Science* **274**, 546–567 (1996).
- Wood, V. et al. The genome sequence of *Schizosaccharomyces pombe*. *Nature* **415**, 871–880 (2002).
- The *C. elegans* Sequencing Consortium Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science* **282**, 2012–2018 (1998).
- Adams, M. D. et al. The genome sequence of *Drosophila melanogaster*. *Science* **287**, 2185–2195 (2000).
- The Arabidopsis Genome Initiative. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408**, 796–815 (2000).
- Lander, E. S. et al. Initial sequencing and analysis of the human genome. *Nature* **409**, 860–921 (2001).
- Baldauf, S. L., Roger, A. J., Wenk-Siefert, I. & Doolittle, W. F. A kingdom-level phylogeny of eukaryotes based on combined protein data. *Science* **290**, 972–977 (2000).
- Loomis, W. F. Genetic networks that regulate development in *Dictyostelium* cells. *Microbiol. Rev.* **60**, 135–150 (1996).
- Eichinger, L., Lee, S. S. & Schleicher, M. *Dictyostelium* as model system for studies of the actin cytoskeleton by molecular genetics. *Microsc. Res. Technol.* **47**, 124–134 (1999).
- Parent, C. A. & Devreotes, P. N. A cell's sense of direction. *Science* **284**, 765–770 (1999).
- Firtel, R. A. & Meili, R. *Dictyostelium*: a model for regulated cell movement during morphogenesis. *Curr. Opin. Genet. Dev.* **10**, 421–427 (2000).
- Noegel, A. A. & Schleicher, M. The actin cytoskeleton of *Dictyostelium*: a story told by mutants. *J. Cell Sci.* **113**, 759–766 (2000).
- Kay, R. R. & Williams, J. G. The *Dictyostelium* genome project: an invitation to species hopping. *Trends Genet.* **15**, 294–297 (1999).
- Cox, E. C., Vocke, C. D., Walter, S., Gregg, K. Y. & Bain, E. S. Electrophoretic karyotype for *Dictyostelium discoideum*. *Proc. Natl Acad. Sci. USA* **87**, 8247–8251 (1990).
- Loomis, W. F. & Kuspa, A. *Dictyostelium—A Model System for Cell and Developmental Biology* (eds Maeda, Y., Inouye, K. & Takeuchi, I.) 15–30 (Universal Academic, Tokyo, 1997).
- Gardner, M. J. et al. Chromosome 2 sequence of the human malaria parasite *Plasmodium falciparum*. *Science* **282**, 1126–1132 (1998).
- Bowman, S. et al. The complete nucleotide sequence of chromosome 3 of *Plasmodium falciparum*. *Nature* **400**, 532–538 (1999).
- Glöckner, G. Large scale sequencing and analysis of AT rich eukaryote genomes. *Curr. Genom.* **1**, 289–299 (2000).

19. Glöckner, G. *et al.* The complex repeats of *Dictyostelium discoideum*. *Genome Res.* **11**, 585–594 (2001).
20. Dear, P. H. in *Genome Mapping—A Practical Approach* (ed. Dear, P. H.) 95–124 (IRL Press, Oxford, 1997).
21. Pan, W. C. & Blackburn, E. H. Single extrachromosomal ribosomal RNA gene copies are synthesized during amplification of the rDNA in *Tetrahymena*. *Cell* **23**, 459–466 (1981).
22. Lim, L. P. & Burge, C. B. A computational analysis of sequence features involved in recognition of short introns. *Proc. Natl Acad. Sci. USA* **98**, 11193–11198 (2001).
23. Morio, T. *et al.* The *Dictyostelium* developmental cDNA project: generation and analysis of expressed sequence tags from the first-finger stage of development. *DNA Res.* **5**, 335–340 (1998).
24. Apweiler, R. *et al.* InterPro—an integrated documentation resource for protein families, domains and functional sites. *Bioinformatics* **16**, 1145–1150 (2000).
25. Goebel, M. G. & Petes, T. D. Most of the yeast genomic sequences are not essential for cell growth and division. *Cell* **46**, 983–992 (1986).
26. Konfortov, B. A., Cohen, H. M., Bankier, A. T. & Dear, P. H. A high-resolution HAPPY map of *Dictyostelium discoideum* chromosome 6. *Genome Res.* **10**, 1737–1742 (2000).
27. Lowe, T. M. & Eddy, S. R. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res.* **25**, 955–964 (1997).
28. Parra, G., Blanco, E. & Guigo, R. GeneID in *Drosophila*. *Genome Res.* **10**, 511–515 (2000).
29. The Gene Ontology Consortium. Gene ontology: tool for the unification of biology. *Nature Genet.* **25**, 25–29 (2000).
30. Fortini, M. E., Skupski, M. P., Boguski, M. S. & Hariharan, I. K. A survey of human disease gene counterparts in the *Drosophila* genome. *J. Cell Biol.* **150**, F23–F30 (2000).

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Competing interests statement

The authors declare that they have no competing financial interests.

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Intracellular calcium stores regulate activity-dependent neuropeptide release from dendrites

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Information in neurons flows from synapses, through the dendrites and cell body (soma), and, finally, along the axon as spikes of electrical activity that will ultimately release neurotransmitters from the nerve terminals. However, the dendrites of many neurons also have a secretory role, transmitting information back to afferent nerve terminals^{1–4}. In some central nervous system neurons, spikes that originate at the soma can travel along dendrites as well as axons, and may thus elicit secretion from both compartments¹. Here, we show that in hypothalamic oxytocin neurons, agents that mobilize intracellular Ca²⁺ induce oxytocin release from dendrites without increasing the electrical activity of the cell body, and without inducing secretion from the nerve terminals. Conversely, electrical activity in the cell bodies can cause the secretion of oxytocin from nerve terminals with little or no release from the dendrites. Finally, mobilization of intracellular Ca²⁺ can also prime the releasable pool of oxytocin in the dendrites. This priming action makes dendritic oxytocin available for release in response to subsequent spike activity. Priming persists for a prolonged period, changing the nature of interactions between oxytocin neurons and their neighbours.

Neurons in the supraoptic nucleus (SON) of the hypothalamus project axons to the posterior pituitary, where oxytocin and vasopressin are secreted from axonal nerve terminals into the systemic circulation. These peptides are also released in large amounts from dendrites in the SON⁵, but secretion at these two sites is not consistently correlated. Suckling evokes oxytocin release in the SON⁶ before significant peripheral secretion, whereas after osmotic stimulation, SON oxytocin release lags behind peripheral secretion⁷. During lactation, in response to suckling, oxytocin cells discharge with brief, intense bursts⁸; these bursts release boluses of oxytocin into the circulation that result in milk let-down from the mammary glands. The bursting activity can be blocked by central administration of oxytocin antagonists⁹, thus central as well as peripheral oxytocin is essential for milk let-down. It has been proposed that suckling evokes dendritic oxytocin release that acts in a positive feedback manner to evoke bursting¹⁰.

Oxytocin mobilizes intracellular Ca²⁺ from thapsigargin-sensitive stores in oxytocin cells¹¹. Here we tested the hypothesis that this might be critical for dendritic oxytocin release. In anaesthetized rats, we implanted a microdialysis probe into the SON to measure oxytocin release in response to systemic osmotic stimulation. In some of these experiments we applied thapsigargin directly to the SON through the dialysis probe. Thapsigargin caused a significant increase in SON oxytocin release that returned to control levels after washout. Subsequent systemic osmotic stimulation (2 ml of 1.5 M NaCl, intraperitoneal injection) caused a much larger release of oxytocin in thapsigargin-pretreated rats than in controls. Osmotically stimulated oxytocin secretion into the circulation was unaffected by exposure of one SON to thapsigargin (Fig. 1a, b).

To test whether thapsigargin potentiated spike-dependent release

from the SON, we placed a microdialysis loop on the ventral surface of the SON, where the dendrites form a dense mat¹². We applied electrical stimuli to the neural stalk to evoke antidromically propagated action potentials in supraoptic neurons. Stimulation produced a large release of oxytocin from the SON in thapsigargin-pretreated rats (Fig. 1c), but not in control rats, despite causing a large secretion of oxytocin into the plasma (data not shown). Thus exposure to thapsigargin appears to 'prime' activity-dependent oxytocin release from dendrites.

To test whether this priming action was specific to dendrites, we studied oxytocin release from isolated neural lobes and SON *in vitro*. Oxytocin secretion from the neural lobe, evoked by depolarization with high K^+ solutions, was unaffected by thapsigargin, caffeine and ryanodine, all of which can release Ca^{2+} from intracellular stores^{11,13} (data not shown). By contrast, thapsigargin caused significant oxytocin release from the SON (Fig. 2a–d), whereas neither caffeine nor ryanodine had any appreciable effect on dendritic release (Fig. 2e–g). Furthermore, thapsigargin (but not caffeine or ryanodine) produced a marked potentiation of subsequent K^+ -induced release ($P < 0.01$, paired t -tests; Fig. 2b–d). No potentiation was seen in response to high K^+ administered 5 min after exposure to thapsigargin (Fig. 2a), but large potentiation was observed in response to high K^+ administered 30, 60 or 90 min after thapsigargin (Fig. 2b–d). Application of an oxytocin agonist also induced significant oxytocin release from the SON ($P < 0.01$,

paired t -tests), as shown previously¹⁴, and, like thapsigargin, potentiated subsequent depolarization-evoked oxytocin release (Fig. 2h).

To investigate the physiological significance of priming of activity-dependent dendritic release, we recorded from single oxytocin neurons in virgin female rats while dialysing the SON with thapsigargin. Thapsigargin had no effect on the mean electrical discharge rate of cells (mean change in firing rate after 30 min exposure, -0.4 ± 0.4 spikes s^{-1} , $n = 7$), but statistical analysis revealed a subtle but important effect on discharge patterning. In oxytocin cells under control conditions, interspike intervals that are much shorter than the average interval tend to be followed by interspike intervals that are longer than average, reflecting the summing effects of slow post-spike hyperpolarization¹⁵. However, in some cells after thapsigargin treatment, intervals much shorter than average were followed by intervals that were also significantly shorter than average. This suggested that the normal post-spike hyperpolarization may be being over-ridden by post-spike depolarization, and hence that activity-dependent exocytosis from the dendrites may have a positive feedback effect on electrical activity.

If oxytocin (and/or other substances) released from dendrites affect the activity of neighbouring cells, then this should be particularly apparent from the effects of 'constant collision' stimulation (CCS, Fig. 3a)¹⁶. This protocol involves activating the neighbours of a recorded cell synchronously in an activity-dependent manner, mimicking the co-ordination of neuronal activity that precedes reflex milk ejection¹⁷. Electrical stimulus pulses applied to the neural stalk evoke antidromic spikes in all supraoptic neurons, except when a spike has occurred in the 10 ms before stimulation, in which case the antidromic spike evoked in that cell will be extinguished by collision with the descending spontaneous spike. During recording of each cell, stimuli (CCS) were applied to the neural stalk 5 ms after every spontaneous spike. Thus the CCS does not affect the

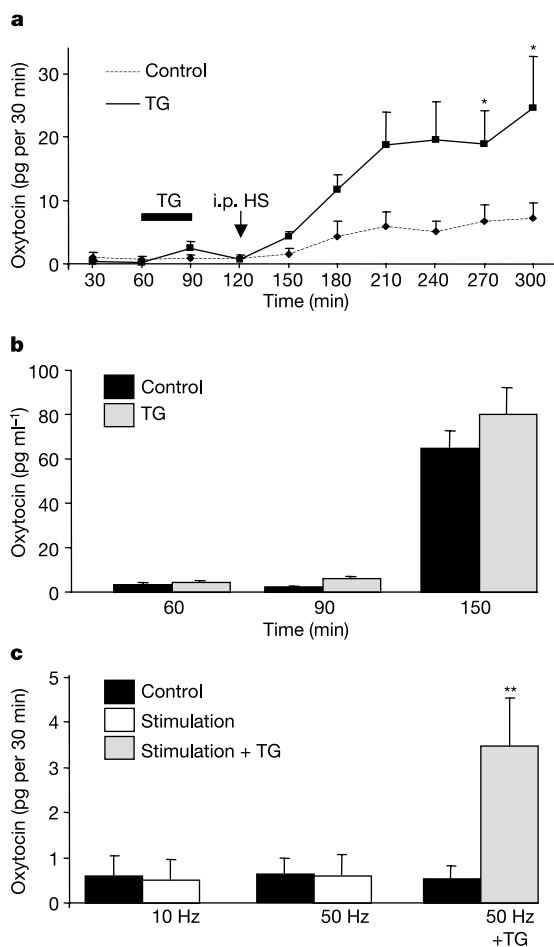


Figure 1 Oxytocin release in the SON. **a**, Release after intraperitoneal (i.p.) injection of hypertonic saline (HS) after dialysis of the SON with thapsigargin (TG) or control solution ($n = 6$). Asterisk, $P < 0.05$, analysis of variance (ANOVA). **b**, Oxytocin measured in the plasma in the same experiments as **a**. **c**, Oxytocin release in the SON before (filled bars) and after electrical stimulation of the neural stalk in control conditions and after exposure of the SON (unilaterally) to thapsigargin ($n = 8$). Double asterisk, $P < 0.01$, t -tests.

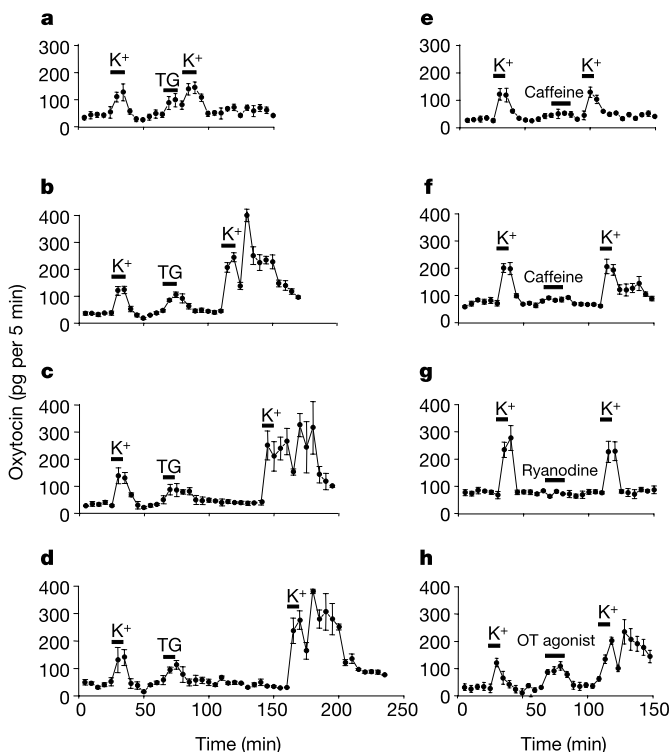


Figure 2 Effects of intracellular calcium mobilizers (horizontal bars) on oxytocin release *in vitro*. **a–g**, Secretion from isolated SON evoked by depolarization with high K^+ solutions was strongly potentiated by thapsigargin in a long lasting, time-dependent manner (**a–d**); by contrast, caffeine (**e**), ryanodine (**g**) had negligible effects. **h**, Oxytocin release from the SON was also potentiated by exposure to an oxytocin (OT) agonist. Means $\pm$ standard error are shown; $n = 4$ per group for each experiment.

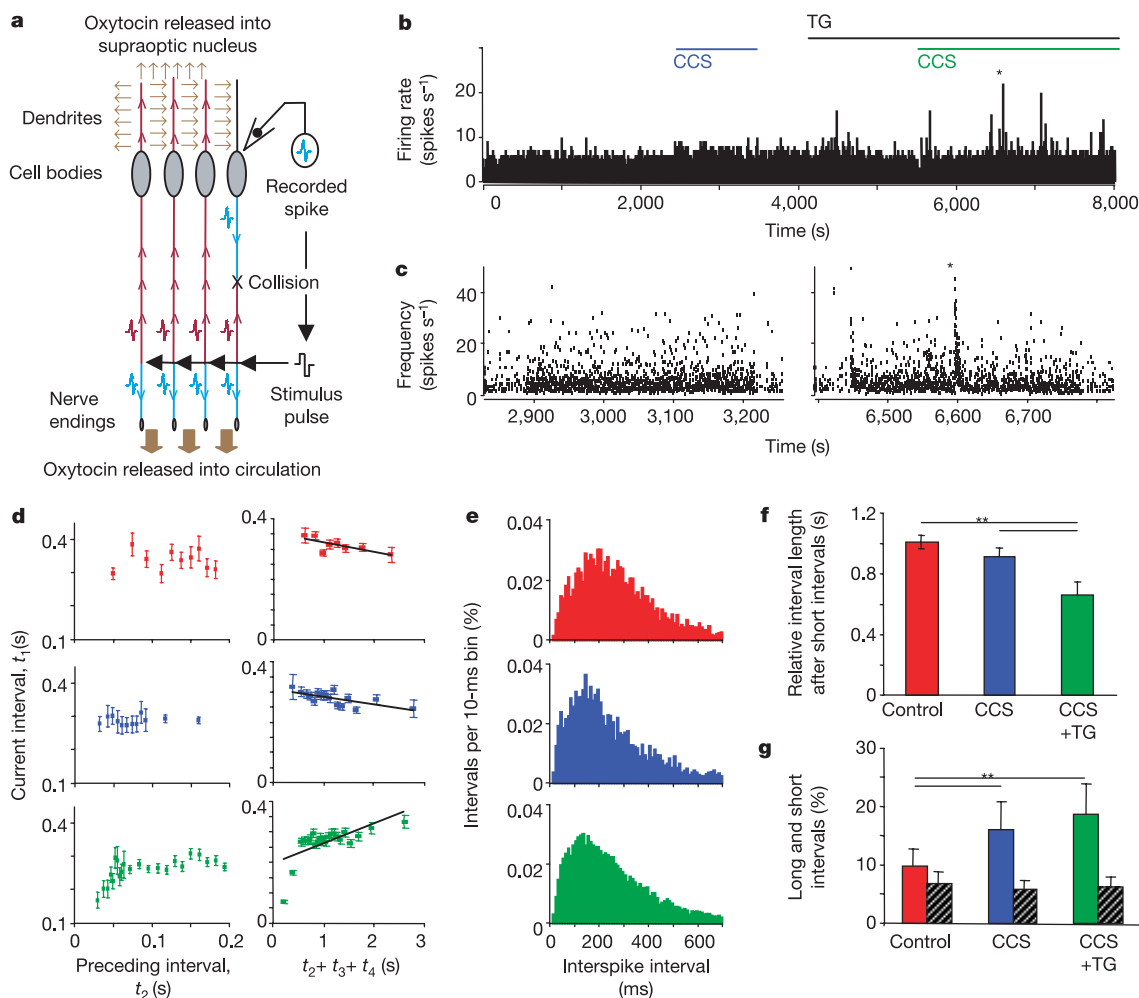


Figure 3 The effects of CCS (**a**) on a representative oxytocin cell (**b–e**), and data from 11 cells exposed to CCS after thapsigargin (**f, g**). After thapsigargin (administered by retrodialysis to the SON), CCS produced clustering of activity with occasional bursts (asterisks in **b, c**). **b–d**, Second-by-second firing rate (**b**), instantaneous frequency (**c**), and mean t_1 ($\pm$ standard error) against t_2 and $t_2 + t_3 + t_4$ (with linear trend lines) for this cell in control conditions (red), during CCS (blue), and during CCS after thapsigargin treatment (green) (**d**) are shown. **e**, Corresponding interspike interval distributions. **f, g**, Data from 11 cells exposed to CCS after thapsigargin (means $\pm$ standard error).

Panel **f** shows the mean t_2 when following short values of t_1 , compared to the overall mean t_2 for each cell. The bars show this ratio in control conditions (red), during CCS (blue), and during CCS after thapsigargin (green). **g**, Mean relative incidence of short intervals (less than 50 ms, hatched bars) and long intervals (greater than 700 ms, coloured bars) in the interspike interval distributions recorded in control conditions, during CCS and during CCS after thapsigargin. Double asterisk, $P < 0.01$, paired t -tests.

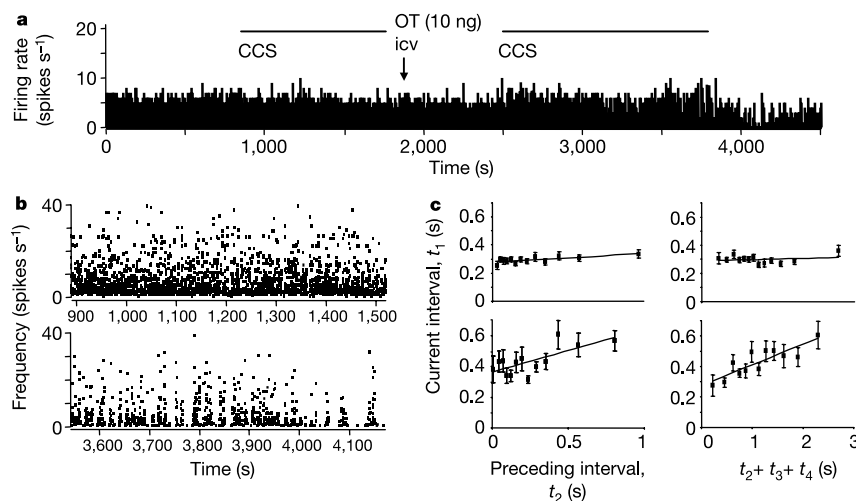


Figure 4 The effects of CCS on a representative oxytocin cell. After oxytocin microinjection (10 ng i.c.v.), CCS produced clustering of activity with occasional bursts. **a–c**, Second-by-second firing rate (**a**), instantaneous frequency (**b**) and mean t_1

($\pm$ standard error) against t_2 , and $t_2 + t_3 + t_4$ (with linear trend lines) for this cell during CCS before (top panels) and after oxytocin injection (bottom panels) (**c**) are shown.

recorded cell directly, as all spikes evoked antidromically in that cell are extinguished; however, antidromic spikes cause approximately synchronous activation of neighbouring cells, and hence will cause synchronized activity-dependent exocytosis from neighbouring dendrites. CCS induces clustered discharge in some oxytocin cells with little effect on the mean discharge rate¹⁶. We compared current and preceding interspike intervals to look specifically for positive feedback effects. During CCS, 7 out of 11 cells showed a positive correlation between the current interspike interval and preceding interval lengths for the shortest intervals (Fig. 3). After thapsigargin treatment, positive correlations were stronger in each of the seven cells during CCS, and positive correlations were seen in the four cells that had not displayed them before thapsigargin treatment (Fig. 3d).

Overall, there was no change in the relative incidence of short intervals (less than 50 ms) in the cells tested with CCS. On the contrary, there was an increase in the incidence of long interspike intervals (greater than 700 ms) (Fig. 3g). Thus, during CCS after thapsigargin treatment, there is no increase in the firing rate of oxytocin cells, and no generally increased incidence of short intervals, but there is a re-organization of spike activity reflected by changes in the interspike interval distributions (Fig. 3e). Very short intervals tend to be followed by short intervals (Fig. 3f) in clusters of spikes that are followed by long intervals, and hence the second-by-second firing rate shows greater variability, as observed before suckling-induced bursts of milk ejection¹⁷. Some cells displayed clear bursts of activity¹⁸ (Fig. 3b, c). These bursts were less intense than 'classical' milk-ejection bursts, which are characterized by extremely short interspike intervals (less than 10 ms) never normally seen in spontaneous activity¹⁹, but were similar to bursts seen at the beginnings of reflex milk ejection²⁰.

In lactating rats, microinjections of oxytocin intracerebroventricularly (i.c.v.) or directly into the SON facilitate the suckling-induced milk ejection reflex for about 30 min⁹. In four experiments we applied CCS to oxytocin cells before and after i.c.v. injection of 10 ng oxytocin into the lateral ventricle. For three of the four cells tested, the effects of oxytocin on spike patterning during CCS were similar to those observed with thapsigargin (Fig. 4); the fourth cell tested was unaffected by oxytocin.

Thus mobilization of Ca^{2+} from thapsigargin-sensitive stores can evoke release of oxytocin from dendrites without an increase of electrical discharge rate and without triggering secretion from axon terminals. This may be a general neuronal mechanism: dendritic secretion of peptides and neurotransmitters has been described for many systems^{2-4,21}, and activity-dependent release of nerve growth factor from hippocampal neurons²² is directly modulated by Ca^{2+} released from intracellular stores. Oxytocin itself mobilizes Ca^{2+} from thapsigargin-sensitive stores in oxytocin cells¹¹, and so, once triggered, oxytocin release may be self-sustaining and hence long lasting.

In addition, we show here that mobilization of intracellular Ca^{2+} is followed by a long-lasting potentiation (priming) of activity- or depolarization-dependent dendritic release. In oxytocin cells, thapsigargin produces a large rise in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), but this effect is transient; $[\text{Ca}^{2+}]_i$ is elevated for only 5–10 min¹¹. Hence the priming is not a consequence of long-lasting elevation of $[\text{Ca}^{2+}]_i$. Furthermore, the effect of thapsigargin is irreversible—the intracellular Ca^{2+} pools depleted by thapsigargin do not refill²³, thus priming cannot reflect a potentiation of intracellular Ca^{2+} release. Priming does not follow an increase in $[\text{Ca}^{2+}]_i$ *per se*, as no priming is induced by exposure to high K^+ alone. The depolarization-evoked rise in $[\text{Ca}^{2+}]_i$ is similar in magnitude to that following thapsigargin treatment¹¹, although it arises from Ca^{2+} entry through voltage-gated channels rather than from intracellular stores. It seems likely that Ca^{2+} release from intracellular stores recruits dendritic granules into a 'readily releasable pool', where they remain available for activity-dependent release for a prolonged period.

After priming, activity-dependent dendritic release facilitates 'clustered' discharge patterning in oxytocin cells. Whether this is a direct action on oxytocin cells, or involves modulation of presynaptic neurotransmitter release²⁴ or actions on interneurons²⁵, is not determined. However, it seems probable that this mechanism underlies the oxytocin-dependent generation of bursting activity in response to suckling. □

Methods

Microdialysis

Adult female rats were anaesthetized with ethyl carbamate (1.25 g kg⁻¹ intraperitoneally). The pituitary stalk and SON were exposed transpharyngeally²⁶. A U-shaped dialysis probe was positioned with the loop of the membrane flat on the ventral surface of the SON¹². Oxytocin was measured by radio-immunoassay in fractions of microdialysate collected every 30 min⁷. In stimulation experiments, an electrode placed on the neural stalk¹⁶ delivered phasic (1 mA, 2-ms matched biphasic pulses; 50 Hz for 3 s/10 s) or continuous stimulation (10 Hz) for 30 min. Blood samples were taken for measurement of oxytocin 5 min before and at the end of stimulation.

Oxytocin release from SON and neural lobes

Female rats (100–200 g) were decapitated, and the SON and neural lobe incubated in oxygenated Locke buffer (four SON or one lobe per well). Drugs (50 mM K⁺, 200 mM thapsigargin, 20 mM caffeine, 2 μM ryanodine, 1 μM oxytocin agonist [Thr4,Gly7]oxytocin; Sigma) were applied for 10–15 min. Oxytocin was measured in 5-min samples of the incubate²⁷.

Electrophysiology

In anaesthetized rats, the activity of antidromically identified SON neurons was recorded, and vasopressin neurons and oxytocin neurons were distinguished by functional and electrophysiological criteria¹². Artificial cerebrospinal fluid (ACSF) was dialysed at 3 $\mu\text{l min}^{-1}$. During recording, dialysis fluid was changed to ACSF containing thapsigargin (0.2 mM, Sigma). Cells were tested with CCS¹⁶ before and after exposure to thapsigargin (applied by retrodialysis) or oxytocin (applied i.c.v.). Interspike interval histograms were constructed in each condition, and scaled to the total number of intervals recorded. Spike trains were analysed for serial dependence. In each period analysed, every interval t_1 was paired with its predecessor t_2 and the preceding trains ($t_2 + t_3 \dots t_n$). The arrays were sorted by ascending t_1 , and binned (bin size 50 for the shortest values of t_1 ; bin size 250 thereafter). For each bin, the corresponding means (+ standard error) of t_2 and ($t_2 + t_3 \dots t_n$) were calculated.

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- Hausser, M., Stuart, G., Racca, C. & Sakmann, B. Axonal initiation and active dendritic propagation of action potentials in substantia nigra neurons. *Neuron* **15**, 637–647 (1995).
- Zilberter, Y., Kaiser, K. M. M. & Sakmann, B. Dendritic GABA release depresses excitatory transmission between layer 2/3 pyramidal and bitufted neurons in rat neocortex. *Neuron* **24**, 979–988 (1999).
- Isaacson, J. S. & Strowbridge, B. W. Olfactory reciprocal synapses—dendritic signalling in the CNS. *Neuron* **20**, 749–761 (1998).
- Isaacson, J. S. Mechanisms governing dendritic γ -aminobutyric acid (GABA) release in the rat olfactory bulb. *Proc. Natl Acad. Sci. USA* **98**, 337–342 (2001).
- Pow, D. V. & Morris, J. F. Dendrites of hypothalamic magnocellular neurons release neurohypophyseal peptides by exocytosis. *Neuroscience* **32**, 435–439 (1989).
- Moos, E. et al. Release of oxytocin within the supraoptic nucleus during the milk ejection reflex in rats. *Exp. Brain Res.* **76**, 593–602 (1989).
- Ludwig, M. Dendritic release of vasopressin and oxytocin. *J. Neuroendocrinol.* **10**, 881–896 (1998).
- Lincoln, D. W. & Wakerley, J. B. Electrophysiological evidence for the activation of supraoptic neurones during the release of oxytocin. *J. Physiol.* **242**, 533–554 (1974).
- Lambert, R. C., Moos, F. C. & Richard, P. Action of endogenous oxytocin within the paraventricular or supraoptic nuclei: a powerful link in the regulation of the bursting pattern of oxytocin neurons during the milk-ejection reflex in rats. *Neuroscience* **57**, 1027–1038 (1993).
- Leng, G., Brown, C. H. & Russell, J. A. Physiological pathways regulating the activity of magnocellular neurosecretory cells. *Prog. Neurobiol.* **57**, 625–655 (1999).
- Lambert, R. C., Dayanithi, G., Moos, F. C. & Richard, P. A rise in the intracellular Ca^{2+} concentration of isolated rat supraoptic cells in response to oxytocin. *J. Physiol.* **478**, 275–288 (1994).
- Ludwig, M. & Leng, G. Autoinhibition of supraoptic nucleus vasopressin neurons *in vivo*—a combined retrodialysis/electrophysiological study in rats. *Eur. J. Neurosci.* **9**, 2532–2540 (1997).
- Berridge, M. J., Bootman, M. D. & Lipp, P. Calcium—a life and death signal. *Nature* **395**, 645–648 (1998).
- Moos, E. et al. Release of oxytocin and vasopressin by magnocellular nuclei *in vitro*: specific facilitatory effect of oxytocin on its own release. *J. Endocrinol.* **102**, 63–72 (1984).
- Kirkpatrick, K. & Bourque, C. W. Activity dependence and functional role of the apamin-sensitive K^+ current in the rat supraoptic neurones *in vitro*. *J. Physiol.* **494**, 389–398 (1996).
- Leng, G. The effects of neural stalk stimulation upon firing patterns in rat supraoptic neurones. *Exp. Brain Res.* **41**, 135–145 (1981).
- Brown, D. & Moos, F. Onset of bursting in oxytocin cells in suckled rats. *J. Physiol.* **503**, 625–634 (1997).
- Lincoln, D. W. & Wakerley, J. B. Accelerated discharge of paraventricular neurosecretory cells correlated with reflex release of oxytocin during suckling. *J. Physiol.* **111**, 327–327 (1972).
- Dyball, R. E. & Leng, G. Regulation of the milk ejection reflex in the rat. *J. Physiol.* **380**, 239–256 (1986).
- Belin, V. & Moos, F. Paired recordings from supraoptic and paraventricular oxytocin cells in suckled rats: recruitment and synchronization. *J. Physiol.* **377**, 369–390 (1986).
- Cheramy, A., Leviet, V. & Glowinski, J. Dendritic release of dopamine in the substantia nigra. *Nature* **289**, 537–542 (1981).
- Blöchl, A. & Thoenen, H. Localization of cellular-storage compartments and sites of constitutive and

- activity-dependent release of nerve growth factor in primary cultures of hippocampal neurons. *Mol. Cell. Neurosci.* **7**, 173–190 (1996).
23. Treiman, M., Caspersen, C. & Christensen, S. B. A tool coming to age: thapsigargin as an inhibitor of sarco-endoplasmic reticulum Ca^{2+} -ATPases. *Trends Pharmacol. Sci.* **19**, 131–135 (1998).
24. Kombian, S. B., Mougnot, D. & Pittman, Q. J. Dendritic released peptides act as retrograde modulators of afferent excitation in the supraoptic nucleus *in vitro*. *Neuron* **19**, 903–912 (1997).
25. Jourdain, P. *et al.* Evidence for a hypothalamic oxytocin-sensitive pattern-generating network governing oxytocin neurons *in vitro*. *J. Neurosci.* **18**, 6641–6649 (1998).
26. Leng, G. & Dyball, R. E. J. in *Neuroendocrine Research Methods* (ed. Greenstein, B.) 769–791 (Harwood GmbH, Switzerland, 1991).
27. Chevalyere, V., Dayanithi, G., Moos, F. C. & Desarmenien, M. G. Developmental regulation of a local positive autocontrol of supraoptic neurons. *J. Neurosci.* **20**, 5813–5819 (2000).

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Competing interests statement

The authors declare that they have no competing financial interests.

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Capacitance steps and fusion pores of small and large-dense-core vesicles in nerve terminals

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The vesicles that package neurotransmitters fall into two distinct classes, large dense-core vesicles (LDCVs) and small synaptic vesicles, the coexistence of which is widespread in nerve terminals¹. High resolution capacitance recording reveals unitary steps proportional to vesicle size. Measurements of capacitance steps during LDCV and secretory granule fusion in endocrine and immune cells have provided important insights into exocytosis^{2–4}; however, extending these measurements to small synaptic vesicles has proven difficult. Here we report single vesicle capacitance steps in posterior pituitary nerve terminals. These nerve terminals contain neuropeptide-laden LDCVs, as well as microvesicles. Microvesicles are similar to synaptic vesicles in size, morphology⁵ and molecular composition^{6–8}, but their contents are unknown. Capacitance steps of two characteristic sizes, corresponding with microvesicles and LDCVs, were detected in patches of nerve terminal membrane. Both types of vesicles fuse in response to depolarization-induced Ca^{2+} entry. Both undergo a reversible fusion process commonly referred to as ‘kiss-and-run’, but only rarely. Fusion pores seen during microvesicle kiss-and-run have a conductance of 19 pS, 11 times smaller than LDCV fusion pores. Thus, LDCVs and microvesicles use structurally different intermediates during exocytosis.

An electron micrograph illustrates the two classes of vesicles described previously in the posterior pituitary³ (Fig. 1a). To detect unitary capacitance steps during microvesicle and LDCV fusion, we improved the method of cell-attached capacitance measurement⁹, reducing the noise level to less than 10 aF root mean square (r.m.s.) (where 1 aF is 10^{-18} farads). Figure 1b, c shows spontaneous upward and downward steps in capacitance, corresponding to the exo- and endocytosis, respectively, of single vesicles. The steps clearly fall into two size groups: the distribution of up-steps has two peaks, one centred at ~50 aF and the other at ~400 aF (Fig. 1d). The average

amplitude in the range 20–120 aF was 67.0 ± 1.7 aF ($N = 235$, from more than 50 patches), and the average amplitude in the range 120–1,500 aF was 412 ± 16 aF ($N = 746$, from more than 50 patches). Assuming spherical geometry, and using a conversion factor of $0.9 \mu\text{F cm}^{-2}$ (ref. 10), we computed the vesicle diameter for each event and averaged these values to obtain 48.5 ± 0.7 nm ($N = 235$) for the small steps and 125 ± 1 nm ($N = 746$) for the large steps. This distribution is consistent with ultrastructural analysis of pituitary nerve terminals showing a mean diameter of 160 nm for LDCVs and ~50 nm for microvesicles (ref. 5). Thus, the two classes of capacitance steps observed here correspond well with the two classes of vesicles seen in nerve terminals (Fig. 1a). Essentially all patches of nerve terminal membrane showed some vesicle activity, with 65% of patches showing both LDCV and microvesicle steps, 25% showing LDCV steps only, and 10% showing microvesicle steps only.

We also examined downward capacitance steps. An amplitude distribution of down-steps not immediately preceded by an up-step (that is, not part of a kiss-and-run sequence, see below) showed that most down-steps were in the microvesicle size range (Fig. 1e). However, the distribution was broader, suggesting an endocytosis pathway into a population of vesicles similar but not identical to the vesicles that undergo exocytosis. Stimulation of the posterior pituitary induces labelling of microvesicles with extracellular markers¹¹, but leaves their overall number unchanged¹². Thus, the endocytosis that rapidly follows exocytosis in this preparation¹³ maintains a nearly constant total number of microvesicles. Large endocytosis steps (greater than 1.5 fF) that we attributed to endosome or vacuole trafficking¹⁴ were also observed, but only rarely (five events).

Membrane depolarization increased the rate of fusion of both LDCVs and microvesicles (Fig. 2). The depolarization method used here—application of 115–130 mM KCl—collapses the K^{+} gradient, and reduces the membrane potential from its resting value of –67 mV to about –20 mV (measured in current clamp). Experiments with the Ca^{2+} -sensitive fluorescent dye fluo-4 showed that this procedure increased intracellular Ca^{2+} concentration from 160 ± 30 nM to 500 ± 180 nM ($N = 5$). This depolarization increased the frequency of capacitance steps, producing an upward staircase (Fig. 2a). Depolarization had no effect in 55% of the patches tested. Some of these patches showed high activity before depolarization, suggesting elevated initial intracellular Ca^{2+} . In other recordings, the depth of the nerve terminal within the tissue may limit KCl access. In patches sensitive to KCl, the distribution of depolarization-induced up-steps included events in the size range for both microvesicles and LDCVs (Fig. 2b). In these patches, the increase in frequency was seen for both small (less than 120 aF) and large (greater than 120 aF) steps (Fig. 2c), indicating that both LDCVs and microvesicles undergo Ca^{2+} -triggered exocytosis. This exocytosis of LDCVs fits with their established role in neuropeptide release; however, Ca^{2+} -triggered exocytosis has not been demonstrated previously for posterior pituitary microvesicles, and the present result suggests that these vesicles have a functional capability similar to that of small synaptic vesicles.

For approximately 5% of the spontaneous up-steps, a down-step with the same amplitude followed within <2 s. These sequential up- and down-steps, referred to as capacitance flickers, had sizes consistent with both LDCVs (Fig. 3a) and microvesicles (Fig. 3b). Flickers were most readily recognized in the absence of depolarization. In depolarized patches the high level of activity made it difficult to associate a down-step unambiguously with a preceding up-step. Capacitance flickers are a common feature in LDCV exocytosis^{2,3}, and have been interpreted as the reversal of an early step in vesicle fusion¹⁵. They can thus be regarded as evidence for kiss-and-run exocytosis¹⁶. The apparent similarity between up- and down-steps was confirmed by analysing the correlation in amplitudes for LDCVs (Fig. 3c) and microvesicles (Fig. 3d). The amplitude of the down-step was tightly correlated with the amplitude of the paired up-step ($P < 0.0001$ for both). By contrast, down-steps

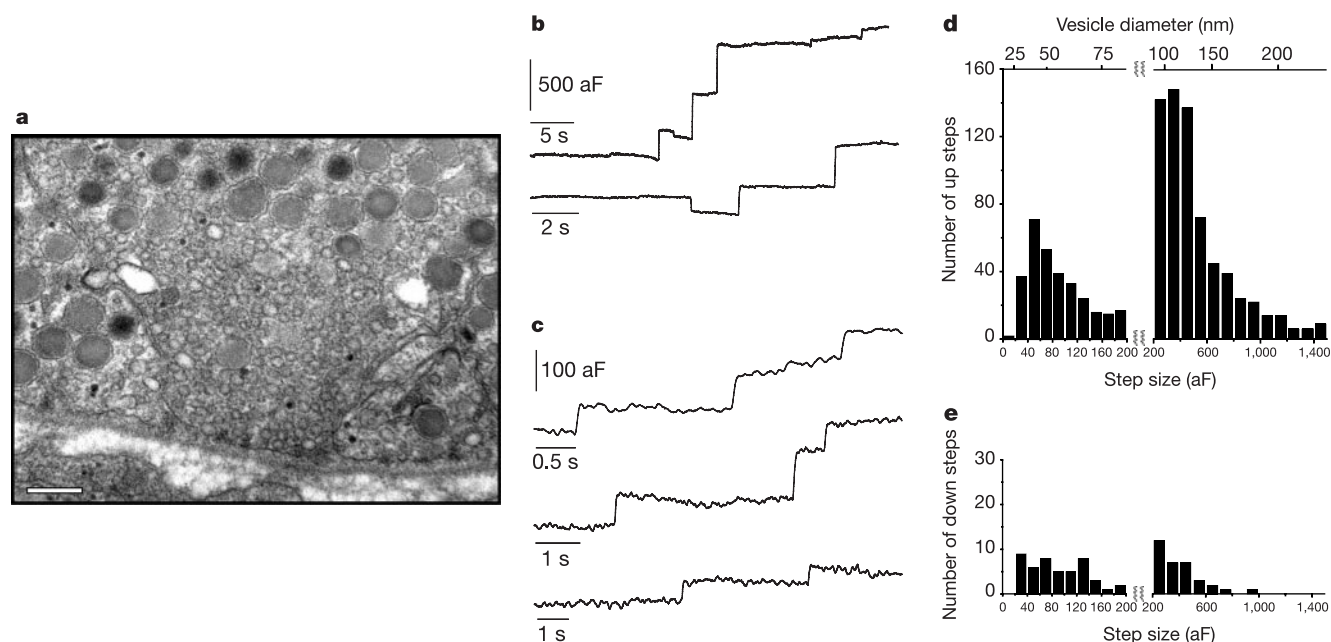


Figure 1 Spontaneous single-vesicle capacitance steps in nerve terminals. **a**, Electron micrograph from a posterior pituitary nerve terminal. The LDCVs are towards the top and a large cluster of microvesicles is below. Scale bar, 200 nm. **b**, Capacitance traces at low scaling show prominent LDCV events as large steps. Smaller microvesicle steps are

evident but less clear at this scale. **c**, Traces scaled up in amplitude show microvesicle fusion events more clearly. **d**, Size distribution of spontaneous upward steps. Numbers of events are plotted in bins of 20 aF (<200 aF) and 100 aF (>200 aF). **e**, Size distribution for spontaneous downward steps; bins as in **d**.

were not correlated with an unpaired up-step occurring earlier or later in the same record ($P > 0.39$ for all). The successive up- and down-steps in Fig. 3a, b therefore represent transient fusion and retrieval of the same vesicle. Thus, both LDCVs and microvesicles undergo kiss-and-run exocytosis.

The plots of up-steps versus paired down-steps in Fig. 3c, d had slopes indistinguishable from unity for both microvesicles and LDCVs, indicating no transport of membrane components between the plasma membrane and vesicle. This is consistent with the

finding that lipid-bound dyes in vesicles fail to move to the plasma membrane while the fusion pore is open^{17,18}.

Kiss-and-run events had different durations for LDCVs (0.56 ± 0.07 s, $N = 39$) and microvesicles (0.31 ± 0.05 s, $N = 29$; $P < 0.05$). During the interval between the up- and down-steps in capacitance, the vesicle lumen has electrical contact with the extracellular fluid. The connection between these two compartments is considered a fusion pore^{19–23}; that is, a gap-junction-like channel presumed to span the vesicle and plasma membranes. The conductances of a variety of fusion pores have been measured, with mean values ranging from 150 to 330 pS (ref. 15). We calculated the conductance of the fusion pores of LDCVs and microvesicles from

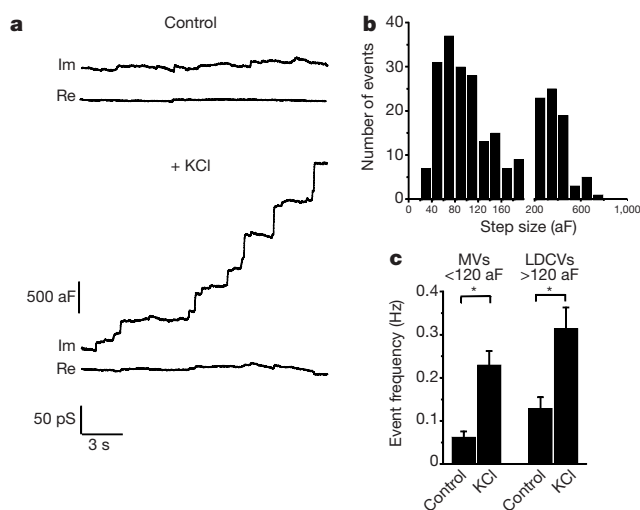


Figure 2 Depolarization-induced Ca^{2+} entry elicits exocytosis of LDCVs and microvesicles. **a**, Capacitance (imaginary) and conductance (real) traces show a low level of spontaneous activity before depolarization (control). Depolarization (+KCl) elicits an upward staircase of capacitance steps, and increased the upward step frequency in 45% of patches tested. **b**, Size distribution of steps elicited by depolarization (binning as in Fig. 1d). **c**, Mean frequencies of small and large capacitance steps from 18 experiments in which depolarization had an effect (asterisk, $P < 0.01$). MVs, microvesicles.

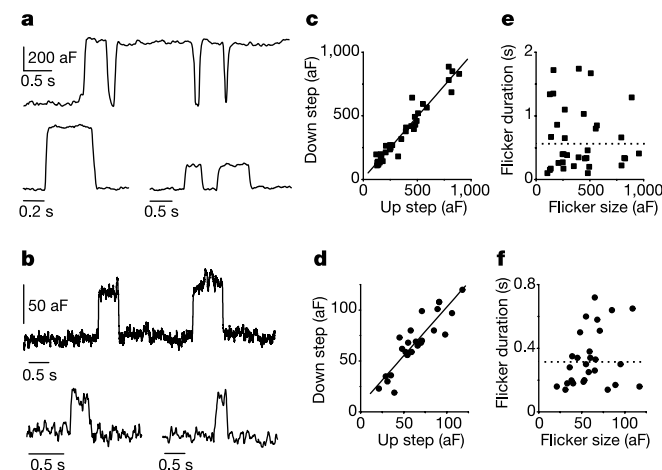


Figure 3 Kiss-and-run. **a**, **b**, Capacitance flickers corresponding to kiss-and-run fusion of LDCVs (**a**) and microvesicles (**b**). **c**, **d**, Plots of downward versus upward steps for LDCVs (**c**) and microvesicles (**d**) show high correlation and slopes near one (0.97 ± 0.04 , $N = 39$ for LDCVs and 0.96 ± 0.1 , $N = 29$ for microvesicles). **e**, **f**, Plot of flicker lifetime versus vesicle size for LDCVs (**e**) and microvesicles (**f**). The mean flicker duration (dotted line) was 0.56 ± 0.07 s for LDCVs and 0.31 ± 0.05 s for microvesicles.

the real (Re) and imaginary (Im) components of the complex admittance (see Methods) using the formula $g = (\text{Re}^2 + \text{Im}^2)/\text{Re}$ (ref. 4). Fusion pore conductance, g , could be measured in 75% of LDCV kiss-and-run events (Fig. 4a), giving a mean value of 213 ± 37 pS (range 23–780 pS, $N = 29$; all from different patches) comparable to that estimated in other preparations¹⁵. By contrast, the conductance of the microvesicle fusion pore (Fig. 4b), which could be measured in 50% of kiss-and-run events at 10 kHz and 67% at 50 kHz, was only 19 ± 3 pS (range 4–45 pS, $N = 15$; all from different patches). Fusion pores could also be detected during ~10% of complete (non-reversing) LDCV fusion events (Fig. 4c). These pores had a duration of 0.19 ± 0.05 s and a conductance of 253 ± 42 pS (range 30–950 pS, $N = 34$), comparable to values seen during LDCV kiss-and-run. In non-reversing microvesicle capacitance steps, we could not resolve the initial fusion pore conductance, even with conditions adjusted to optimize resolution. On the basis of our estimated time resolution of 2 ms, we can only conclude that the fusion pore intermediate in non-reversing microvesicle fusion was either too brief, or its conductance too high to detect. Thus, although fusion pores during LDCV kiss-and-run are quite similar to those seen during non-reversing LDCV steps, microvesicle fusion pores may be different in these two situations.

The large difference between LDCV and microvesicle fusion pore conductances suggests that different structures mediate the exocytosis of these two vesicle types. To obtain an estimate of pore diameter from conductance, we used the following formula from ref. 20: $g = \pi r^2/(\rho \lambda)$, where ρ is the saline resistivity (100 Ω cm), λ is the length of a gap junction channel (15 nm), and r is the radius. This yielded $r = 1.0$ nm for LDCVs and $r = 0.3$ nm for microvesicles. Although these estimates are rough, they underscore the point that the conductance difference between LDCV and microvesicle fusion pores implies a significant difference in structure.

The small size of the microvesicle fusion pore is comparable to the size of many neurotransmitter molecules, and would therefore present a substantial barrier to the efflux of neurotransmitter during kiss-and-run fusion. The conductance of the fusion pore can be used to evaluate the time needed for a vesicle to discharge its content as $\tau = V/(\rho Dg)$ (ref. 21), where V is the vesicle volume, ρ is the solution resistivity (100 Ω cm), and D is the diffusion constant of the

neurotransmitter. We calculated V from the microvesicle capacitance assuming spherical geometry. We used the diffusion constant of acetylcholine of 6×10^{-6} cm² s⁻¹ (ref. 24) as an upper bound to D , as hindrance by the walls of the pore would retard diffusion. This makes our estimate of τ a lower bound. For microvesicles we obtained $\tau > 5.7$ ms. Because studies of synaptic transmission indicate that the contents of a vesicle must be released in < 100 μ s to give the observed time course of a miniature synaptic current^{24,25}, this indicates that diffusion of neurotransmitter through a 19 pS fusion pore is far too slow to support synaptic transmission. Kiss-and-run would thus seem to be a very inefficient mechanism of transmitter release. The time required for a microvesicle to discharge its content by diffusion is still considerably shorter than the mean duration of a microvesicle kiss-and-run event (0.31 s). Even with a τ for expulsion that is 20 times longer than our minimum of 5.7 ms, a vesicle will still have time to empty its contents. However, emptying a vesicle at this slow speed is much more likely to desensitize postsynaptic receptors than activate them.

The problem of slow diffusion through a fusion pore was analysed previously²⁵, and that study indicated the need for a mechanism to accelerate transmitter expulsion. Two hypothetical mechanisms, ion interchange²⁶ and expansion of a core polymer gel within a vesicle²⁷, have been proposed. Fluctuations in fusion pore size that are too rapid to resolve with our instrumentation could provide brief intervals for much more rapid efflux than estimated here. Such fluctuations are indicated by simultaneous capacitance and amperometry recording, which revealed that LDCV flickers can terminate with a brief, transient expansion of the fusion pore to much larger diameters²⁸. Furthermore, it is also important to note that in fusion events where a pore could not be detected, the pore conductance might be large enough to support more rapid transmitter expulsion. It has been pointed out that if fusion pores dilate 100 times more rapidly for synaptic vesicles than for LDCVs, transmitter efflux would be sufficiently rapid to produce synaptic currents with the observed time course²⁴. Our finding of an 11-fold difference between microvesicle and LDCV fusion pore conductances makes this hypothesis more plausible. On the basis of our failure to detect pores during irreversible microvesicle fusion, dilation should take less than 2 ms. Fusion pores were not seen in

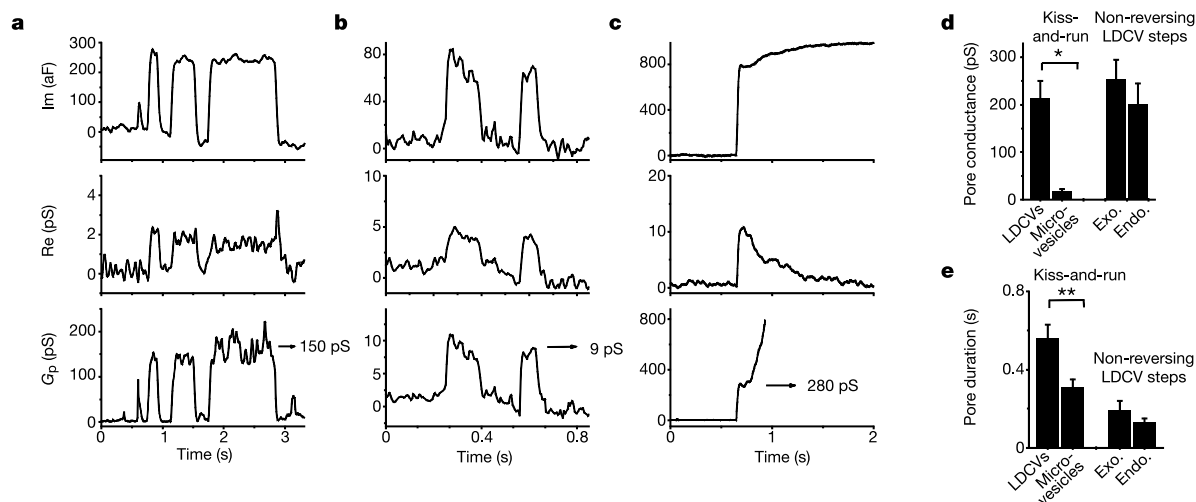


Figure 4 Fusion pore analysis for kiss-and-run and non-reversing events. **a–c**, Imaginary and real traces are used to calculate fusion pore conductance (see Methods) for three LDCV kiss-and-run events (**a**), a pair of microvesicle kiss-and-run events (**b**), and a non-reversing LDCV step (**c**). Lock-in frequency was 10 kHz for these recordings. G_p , fusion pore conductance. **d**, Mean fusion pore conductances for kiss-and-run LDCV and

microvesicle events, and for non-reversing LDCV steps. **e**, Mean fusion pore lifetimes (taken as the width at half-height for flickers and as the time the real trace deviates from baseline for steps) for the same events (asterisk, $P < 0.01$; double asterisk, $P < 0.05$). Exo., exocytosis; Endo., endocytosis.

amperometry recordings of small vesicle fusion events in snail neurons²³, and this may also reflect faster pore dilation. Alternatively, the initial fusion pores in these events could be much larger than those seen here during kiss-and-run, and that too would allow for rapid transmitter efflux.

This study has shown that microvesicles fuse with the plasma membrane of nerve terminals in the same stepwise fashion described previously for LDCVs and secretory granules^{2–4,20}. Both LDCVs and microvesicles undergo exocytosis in response to membrane depolarization, and both undergo kiss-and-run fusion. However, the fusion pores that serve as intermediates in the exocytosis of LDCVs and microvesicles differ markedly in their basic properties. If the fusion pore is a proteinaceous channel¹⁵, this difference suggests that the fusion machinery used by these vesicles has a different molecular composition. This may contribute to the differential release kinetics of various neurotransmitters, as well as differential sensitivities of neurotransmitter release to diverse forms of physiological stimulation²⁹. □

Methods

Pituitary slices

Slices of posterior pituitary (thickness ~75 µm) were cut with a vibratome¹³. Slices were prepared and maintained in physiological saline (in mM) 125 NaCl, 4 KCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂, 10 glucose, bubbled with 95% O₂/5% CO₂.

Electrical recording

Cell-attached capacitance measurements⁹ were performed with an SR830 2-phase lock-in amplifier coupled to an EPC-7 patch-clamp amplifier. A 10–50 kHz, 200 mV r.m.s. sine wave was superimposed on a command potential of 0 mV. The in-phase (real) and 90° out-of-phase (imaginary) outputs of the lock-in amplifier were low-pass filtered at 3 or 10 ms (24 dB), digitized at 16 bits, and read into a Macintosh computer. The patch-clamp amplifier was operated at a gain of 50 mV pA⁻¹ with C_{slow} set to the minimum and G_{slow} set to 0.2 µS. The current output of the EPC-7 was connected to the lock-in amplifier input through a 5:1 voltage divider. In the beginning of each recording the sinusoidal current was nulled using the C_{fast} and τ_{fast} potentiometers. Phase was adjusted to null the changes in the real trace in response to a 100-fF calibration step. Small phase errors were corrected off-line by recomputing the real and imaginary traces with the phase shift varied in 2° increments to eliminate the projection of capacitance steps on the real trace⁹.

Reconstruction of the real trace using the time course of the imaginary trace and true vesicle capacitance gave results similar to the observed real trace, confirming the internal consistency of our analysis of fusion pores (Supplementary Information). The recording solution contained (in mM): 125 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES, pH 7.2. The pipette solution was the same but with 10 mM CaCl₂, 13 mM tetraethylammonium ion (TEA) and no glucose. TEA was added to block K⁺ channels, but as channel openings were infrequent it was often omitted. The resistance of our patch electrodes ranged from 3 to 4.5 MΩ when filled with this solution. Nerve terminals were depolarized by applying a solution consisting of (in mM) 115 or 130 KCl, 10 NaCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, pH 7.2, from a nearby micropipette.

The noise level was reduced by using thick-wall pipettes heavily coated with Sylgard. A critical step for noise reduction was lowering the depth of electrode immersion together with covering the aqueous surface with dimethylpolysiloxane silicone oil³⁰.

Vesicle fusion pores reduce the imaginary signal and produce a correlated increase in the real signal^{4,19}. The fusion pore conductance was calculated from these traces according to the formula: (Im² + Re²)/Re (ref. 9). From the noise levels in our recordings we estimate the detection range for microvesicle fusion pores as 1.5–40 pS at 10 kHz and 8–200 pS at 50 kHz. For LDCVs the lower range is the same and the upper range is a few nS (ref. 9).

Electron microscopy

Electron microscopy was performed with a Philips CM120 transmission electron microscope on tissue fixed in 2.0% paraformaldehyde/2.5% glutaraldehyde for 2 h, postfixed with 2% osmium tetroxide for 1 h (both at 4°C), dehydrated, embedded, and sectioned at 80 nm.

Data analysis

Data analysis was performed with the programs IGOR and Origin. Means are presented ± standard errors. Statistical significance was analysed using a 2-tailed *t*-test. Slow drifts in capacitance traces were removed by linear fitting.

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- De Camilli, P. & Jahn, R. Pathways to regulated exocytosis in neurons. *Ann. Rev. Physiol.* **52**, 625–645 (1990).
- Neher, E. & Marty, A. Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. *Proc. Natl Acad. Sci. USA* **79**, 6712–6716 (1982).
- Fernandez, J. M., Neher, E. & Gomperts, B. D. Capacitance measurements reveal stepwise fusion events in degranulating mast cells. *Nature* **312**, 453–455 (1984).
- Lollike, K., Borregaard, N. & Lindau, M. The exocytotic fusion pore of small granules has a conductance similar to an ion channel. *J. Cell Biol.* **129**, 99–104 (1995).
- Morris, J. F., Nordmann, J. J. & Dyball, R. E. J. Structure-function correlation in mammalian neurosecretion. *Int. Rev. Exp. Pathol.* **18**, 1–95 (1978).
- Navone, F. et al. Microvesicles of the neurohypophysis are biochemically related to small synaptic vesicles of presynaptic nerve terminals. *J. Cell Biol.* **109**, 3425–3433 (1989).
- Walch-Solimena, C. et al. Synaptotagmin: a membrane constituent of neurosecretory-containing large dense-core vesicles. *J. Neurosci.* **13**, 3895–3903 (1993).
- Pupier, S. et al. Cysteine string proteins associated with secretory granules of the rat neurohypophysis. *J. Neurosci.* **17**, 2722–2727 (1997).
- Debus, K. & Lindau, M. Resolution of patch capacitance recordings and of fusion pore conductance in small vesicles. *Biophys. J.* **78**, 2983–2997 (2000).
- Gentet, L. J., Stuart, G. J. & Clements, J. D. Direct measurement of specific membrane capacitance in neurons. *Biophys. J.* **79**, 314–320 (2000).
- Nagasawa, J., Douglas, W. W. & Schulz, R. A. Micropinocytotic origin of coated and smooth microvesicles ('synaptic vesicles') in neurosecretory terminals of posterior pituitary glands demonstrated by incorporation of horse-radish peroxidase. *Nature* **332**, 341–342 (1971).
- Morris, J. F. & Nordmann, J. J. Membrane recapture after hormone release from nerve endings in the neural lobe of the rat pituitary gland. *Neuroscience* **5**, 639–649 (1980).
- Hsu, S.-F. & Jackson, M. B. Rapid exocytosis and endocytosis in nerve terminal of the rat posterior pituitary. *J. Physiol.* **494**, 539–553 (1996).
- Rosenboom, H. & Lindau, M. Exo-endocytosis and closing of the fission pore during endocytosis in single pituitary nerve terminals internally perfused with high calcium concentrations. *Proc. Natl Acad. Sci. USA* **91**, 5267–5271 (1994).
- Lindau, M. & Almers, W. Structure and function of fusion pores in exocytosis and ectoplasmic membrane fusion. *Curr. Opin. Neurobiol.* **7**, 509–517 (1995).
- Fesce, R., Grohovaz, F., Valtorta, F. & Meldolesi, J. Neurotransmitter release: fusion or 'kiss-and-run'? *Trends Cell Biol.* **4**, 1–4 (1994).
- Tse, F. W., Iwata, A. & Almers, W. Membrane flux through the pore formed by a fusogenic viral envelope protein during cell fusion. *J. Cell Biol.* **121**, 543–552 (1993).
- Zimmerberg, J., Blumenthal, R., Curran, M., Sarker, D. & Morris, S. Restricted movement of lipid and aqueous dyes through pores formed by influenza hemagglutinin during cell fusion. *J. Cell Biol.* **127**, 1885–1894 (1994).
- Breckenridge, L. J. & Almers, W. Currents through the fusion pore that forms during exocytosis of a secretory granule. *Nature* **328**, 814–817 (1987).
- Spruce, A. E., Breckenridge, L. J., Lee, A. K. & Almers, W. Properties of the fusion pore that forms during exocytosis of a mast cell secretory granule. *Neuron* **4**, 643–654 (1990).
- Almers, W. et al. Millisecond studies of single membrane fusion events. *Ann. NY Acad. Sci.* **635**, 318–327 (1991).
- Chow, R. H., von Rüden, L. & Neher, E. Delay in vesicle fusion revealed by electrochemical monitoring of single secretory events in adrenal chromaffin cells. *Nature* **356**, 60–63 (1992).
- Bruns, D. & Jahn, R. Real-time measurement of transmitter release from single synaptic vesicles. *Nature* **377**, 62–65 (1995).
- Stiles, J. R., Van Helden, D., Bartol, T. M., Salpeter, E. E. & Salpeter, M. M. Miniature endplate current rise times <100 µs from improved dual recordings can be modeled with passive acetylcholine diffusion from a synaptic vesicle. *Proc. Natl Acad. Sci. USA* **93**, 5747–5752 (1996).
- Khanin, R., Parnas, H. & Segel, L. Diffusion cannot govern the discharge of neurotransmitter in fast synapses. *Biophys. J.* **67**, 966–972 (1994).
- Khanin, R., Parnas, H. & Segel, L. A mechanism for discharge of charged excitatory neurotransmitter. *Biophys. J.* **72**, 507–521 (1997).
- Nanavati, C. & Fernandez, J. M. The secretory granule matrix: a fast acting smart polymer. *Science* **259**, 963–965 (1993).
- Ales, E. et al. High calcium concentrations shift the mode of exocytosis to the kiss-and-run mechanism. *Nature Cell Biol.* **1**, 40–44 (1999).
- Verhage, M. et al. Differential release of amino acids, neuropeptides, and catecholamines from isolated nerve terminals. *Neuron* **6**, 517–524 (1991).
- Rae, J. L. & Levis, R. A. A method for exceptionally low noise single channel recordings. *Pflügers Arch. Eur. J. Physiol.* **420**, 618–620 (1992).

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Competing interests statement

The authors declare that they have no competing financial interests.

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Dynamic interactions of cyclic AMP transients and spontaneous Ca²⁺ spikes

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Transient increases of intracellular Ca²⁺ drive many cellular processes, ranging from membrane channel kinetics to transcriptional regulation^{1–5}, and links of Ca²⁺ to other second messengers should activate signalling networks^{6–11}. However, real-time kinetic interactions have been difficult to investigate. Here we report observations of spontaneous increases in concentration of cyclic AMP (cAMP) in embryonic spinal neurons, and their dynamic interactions with Ca²⁺ oscillations. Blocking the production of these cAMP transients decreases the intrinsic frequency of spontaneous Ca²⁺ spikes, whereas inducing cAMP increases causes spike frequency to increase. Transients of cAMP in turn are absent when Ca²⁺ spikes are blocked, and are generated only in response to specific patterns of stimulated spikes that mimic endogenous Ca²⁺ kinetics. We present a mathematical model of Ca²⁺–cAMP reciprocity that generates the slow cAMP oscillations and reproduces the dynamics of Ca²⁺–cAMP interactions observed experimentally. The model predicts that this module of

coupled second messengers is tuned to optimize production of cAMP transients, and that simultaneous stimulation of Ca²⁺ and cAMP systems produces distinct temporal patterns of oscillations of both messengers. Our findings may prove useful in the investigation of the regulation of gene expression by second-messenger transients.

To investigate the interactions between Ca²⁺ and cAMP second-messenger systems we tested whether embryonic neurons generating spontaneous Ca²⁺ spikes produce increases in the concentration of cAMP. Transients of cAMP were observed at a frequency of $4.3 \pm 1.1 \text{ h}^{-1}$ and duration of $2.9 \pm 0.3 \text{ min}$ in 58% of young neurons ($n = 17$). Because the frequency of spontaneous Ca²⁺ spikes decreases during development³, we also examined cAMP transient generation in mature neurons. cAMP transients were observed in 50% of these cells, with a similar frequency ($3.0 \pm 0.8 \text{ h}^{-1}$, $P > 0.9$) but longer duration ($7.2 \pm 0.8 \text{ min}$, $P < 0.001$) than those of young neurons ($n = 12$; Fig. 1, see Supplementary Information for movie). cAMP increases occurred synchronously throughout neurons, suggesting that they could be driven by rapidly propagated Ca²⁺ spikes. The amplitudes of these spontaneous cAMP transients were comparable to those of cAMP increases induced by 50 μM forskolin, an activator of adenylyl cyclase (AC), in both young and mature neurons.

We next determined whether changes in cAMP levels modulate Ca²⁺ spike frequency. Sustained increases in cAMP produced by application of 50 μM forskolin increased the frequency of spontaneous Ca²⁺ transients in 80% of young neurons from $10.0 \pm 1.2 \text{ h}^{-1}$ to $26.5 \pm 1.4 \text{ h}^{-1}$ ($n = 15$, $P < 0.001$; Fig. 2a). In mature cells the effect was smaller, with a change in frequency from $3.2 \pm 1.1 \text{ h}^{-1}$ to $5.2 \pm 1.5 \text{ h}^{-1}$ in 27% of cells ($n = 11$, $P < 0.03$). Inhibiting AC with 40 μM dideoxyadenosine decreased the frequency of spontaneous Ca²⁺ transients in young neurons from $10.7 \pm 1.8 \text{ h}^{-1}$ to $4.6 \pm 1.5 \text{ h}^{-1}$ in 80% of cells ($n = 10$, $P < 0.08$; Fig. 2b, c), suggesting that endogenous AC activity regulates the

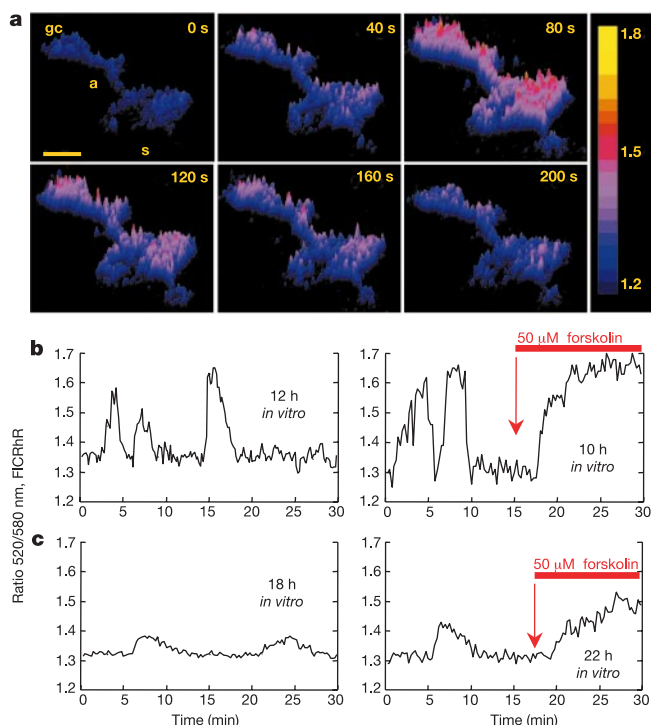


Figure 1 Spontaneous transient increases of cAMP in cultured spinal neurons. **a**, Surface plot images illustrate increases and decreases in ratio fluorescence of FICRHR in a foreshortened view of a young neuron, from the soma (s) along the axon (a) to the growth cone (gc). Colour scale indicates 520 nm/580 nm fluorescence ratio. Scale bar, 10 μm . **b**, Digitized image intensities of the somata of two young neurons; forskolin applied following the transients in the second provides a physiological calibration of the indicator. **c**, As in **b**, for mature neurons.

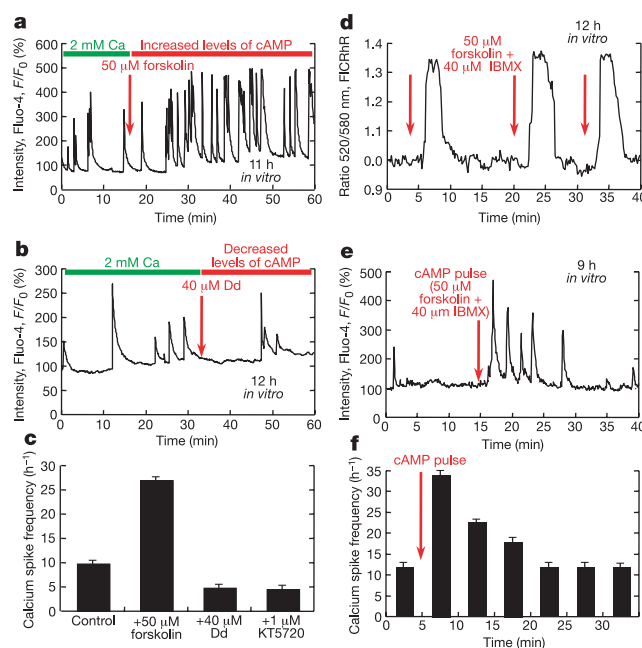


Figure 2 Changes in cAMP levels modulate Ca²⁺ spike frequency. **a**, Sustained increase of cAMP by stimulation with forskolin increases the frequency of Ca²⁺ spikes. **b**, Blockade of spontaneous cAMP transients with dideoxyadenosine (Dd) reduces the frequency of Ca²⁺ spikes. **c**, Histogram of Ca²⁺ spike frequency dependence on steady levels of cAMP ($n \geq 5$). **d**, Increases of cAMP induced with 5-s pulses of saline containing forskolin and IBMX mimic naturally occurring transients. **e**, Phasic increase of cAMP (arrow) increases the frequency of spontaneous Ca²⁺ spikes for a brief period. **f**, Histogram of Ca²⁺ spike frequency before and after the cAMP pulse ($n = 7$).

natural spike frequency. Physiologically relevant imposition of cAMP transients produced by 5-s pulses of a solution containing 50 μM forskolin and 40 μM IBMX (3-isobutyl-1-methylxanthine, to block phosphodiesterase, PDE), mimicking transients generated spontaneously (Fig. 2d), increased Ca^{2+} spike frequency from $12.0 \pm 1.4 \text{ h}^{-1}$ to $34.1 \pm 2.4 \text{ h}^{-1}$ for several minutes in 70% of cells ($n = 10$, $P < 0.03$; Fig. 2e, f). Increases of cAMP may act via protein kinase A (PKA). Consistent with this idea, blocking PKA with 1 μM KT5720 reduced transient frequency to $4.4 \pm 1.7 \text{ h}^{-1}$ in young neurons ($n = 4$ of 5 cells, $P < 0.02$; Fig. 2c).

Are Ca^{2+} spikes necessary to induce changes in cAMP levels? Elimination of naturally occurring Ca^{2+} spikes by the removal of extracellular Ca^{2+} , or by the addition of Ca^{2+} and Na^{+} channel blockers in the presence of Ca^{2+} , blocked production of cAMP transients ($n = 10$), although responses to stimulation with forskolin and IBMX demonstrated that AC remained active ($n = 3$). We then asked whether there is an optimal pattern of Ca^{2+} spikes to stimulate cAMP transients. Ca^{2+} transients were generated in different patterns by depolarizing cells repetitively with brief pulses of 100 mM KCl in the presence of extracellular Ca^{2+} . To reimpose more accurately the frequencies of Ca^{2+} spikes generated spontaneously, we first applied wavelet analysis to recordings from 12 young neurons to calculate the frequency composition. This algorithm probes the local frequency content of the signals, in contrast to Fourier analysis that represents signals by harmonic oscillations extending over the whole time interval, and is less vulnerable to local violations of signal regularity such as sharp transients, noise and shifts in baseline (see Methods and Sup-

plementary Information). All records exhibited three major frequencies. The low frequency ($\sim 4.3 \text{ h}^{-1}$) corresponds to the incidence of bursts and the highest frequency ($\sim 60 \text{ h}^{-1}$) to spikes within bursts, while the intermediate frequency ($\sim 8.2 \text{ h}^{-1}$) represents single spikes (Fig. 3a).

No cAMP increases were observed following single Ca^{2+} transients presented at 10 h^{-1} ($n = 8$; Fig. 3b, e). Triplets of Ca^{2+} transients generated at 4 h^{-1} with an intraburst frequency of 60 h^{-1} elicited cAMP increases in 60% of young neurons ($n = 5$; Fig. 3c, f). Bursts of five Ca^{2+} transients produced at 2 h^{-1} with an intraburst frequency of 60 h^{-1} generated cAMP increases in 75% of young neurons ($n = 4$; Fig. 3d, g). Although the total number of spikes delivered within 1 h for each protocol was close to the spontaneous mean, the pattern of stimulation strongly influenced the amplitude and duration of cAMP responses. Depolarization alone was insufficient, because no cAMP increases were observed when Ca^{2+} was omitted from the saline and cells were depolarized with KCl ($n = 13$). These observations suggest that specific patterns of Ca^{2+} spikes are selectively translated into cAMP transients, whereas other patterns are not.

To identify the constraints on the second-messenger interactions necessary to generate these experimental findings, we used a scheme (Fig. 4a) to construct a model producing intracellular Ca^{2+} oscillations and Ca^{2+} -cAMP interactions. To describe Ca^{2+} dynamics we modified a single-compartment system¹² in which $[\text{Ca}^{2+}]$ is regulated by InsP_3 -receptor-activated stores and Ca^{2+} pumps, although a role for such stores has not yet been demonstrated in the production of spikes in these neurons. The model is represented by the equations:

$$\frac{dx}{dt} = \alpha_1(\beta - x) + \alpha_2 x \frac{(\beta - x)(1 - y)}{(x + \beta_0)} - \beta_1 \frac{x^2}{(x^2 + \alpha_3^2)} + \alpha_4 \frac{z^2}{(z^2 + \alpha_5^2)} \quad (1)$$

$$\frac{dy}{dt} = -\epsilon y + \beta_2 x^2 \frac{(1 - y)}{(x + \beta_0)} \quad (2)$$

where x and y represent the concentrations of Ca^{2+} and InsP_3 receptor (InsP_3R) bound to two Ca^{2+} ; α_i , β_i and ϵ are constants. The last term was added to equation (1) to account for positive feedback on $[\text{Ca}^{2+}]$ induced by increases in cAMP, consistent with PKA-driven phosphorylation of ryanodine and InsP_3 receptors^{13,14}. The dynamics of cAMP were described by a third equation (see below) in which cAMP is produced by Ca^{2+} -activated AC¹⁵ and degraded by cAMP-activated PDE¹⁶. Differences in the kinetics of cAMP generation and removal produce oscillations in response to Ca^{2+} transients.

$$\frac{dz}{dt} = a_1 x^4 - a_2 \frac{z^3}{(z^2 + a_3^2)} \quad (3)$$

where z is the concentration of cAMP and a_i are constants. The strength of feedback interactions and constants for the model were derived from experimental data (see Supplementary Information).

The model produces spontaneous patterns of Ca^{2+} and cAMP oscillations similar to those observed experimentally. First, cAMP transients are generated by Ca^{2+} increases and terminated by self-activated inhibition (Fig. 4b, top and bottom). Although the transients produced by the simulation are periodic, introduction of noise would generate aperiodic behaviour resembling experimental records¹⁷. Second, wavelet analysis of Ca^{2+} transients produced by the model shows strong periodic wavelet coefficients at scales of 80 and 15, identifying dominant frequencies similar to those from analysis of experimental records (Fig. 4b, middle). Third, transient increases of cAMP increase the frequency of Ca^{2+} transients briefly (Fig. 4c). Fourth, Ca^{2+} spikes imposed in different patterns are differentially effective in stimulating increases of cAMP

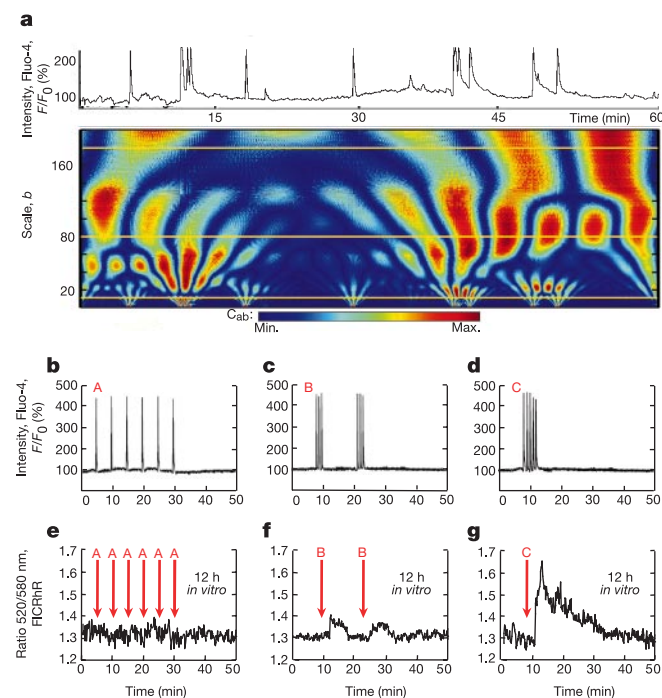


Figure 3 cAMP transients decode specific patterns of Ca^{2+} spikes. **a**, Wavelet analysis identifies prominently represented frequencies of spontaneous Ca^{2+} spikes. Top, digitized record of Ca^{2+} indicator fluorescence from young neuron imaged for 1 h. Bottom, pseudocolour density plot of the wavelet coefficient, C_{ab} , as a function of the scale, b , and time (as for the top panel) for this record. Yellow horizontal lines indicate strong periodic C_{ab} values at specific scales (180, 80 and 12) corresponding to frequencies at which spikes are generated. **b–d**, Different patterns of stimulated Ca^{2+} spikes. A, single spikes; B, three spikes; C, five spikes. **e–g**, cAMP responses to these patterns of Ca^{2+} spike stimulation; red arrows mark the times of imposition of the patterns of Ca^{2+} transients indicated by the letter above each one.

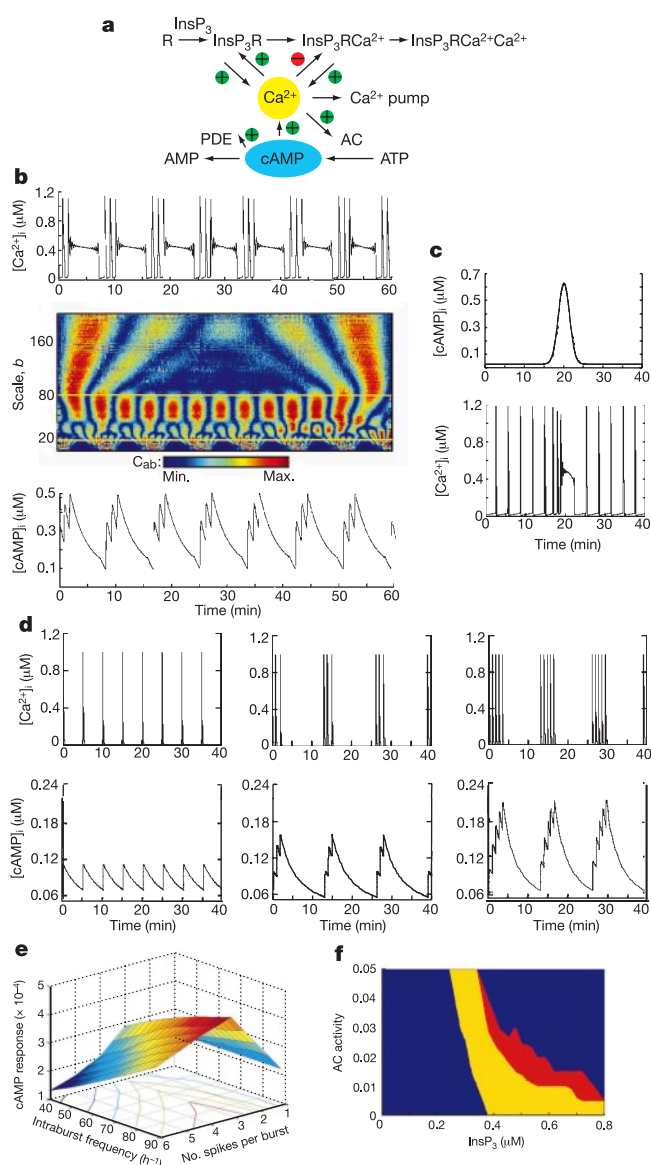


Figure 4 Modelling dynamics of Ca^{2+} –cAMP interactions. **a**, Scheme illustrating the interactions on which the model is based. InsP_3 , inositol trisphosphate; InsP_3R , InsP_3 receptor. **b**, Top and bottom, simulated spontaneous Ca^{2+} and cAMP oscillations. Baseline $[\text{Ca}^{2+}]$ and $[\text{cAMP}]$ are ~ 20 and ~ 200 nM. Middle, wavelet analysis of modelled Ca^{2+} oscillations. Nomenclature as in Fig. 3a. **c**, Simulated transient increase of cAMP increases the frequency of Ca^{2+} spikes transiently. **d**, Different patterns of simulated Ca^{2+} spikes are selectively potent in eliciting cAMP responses. **e**, Variation of spike number (proportional to burst duration) and intraburst spike frequency identifies a maximum for the cAMP response, defined as the ratio of signal amplitude (μM , baseline to peak) to the signal duration (s, baseline to baseline) to provide a measure of sharpness, for triplets of spikes over a range of intraburst frequencies. **f**, Simultaneous increases in $[\text{InsP}_3]$ and AC activity (a_1 in equation (3)) within physiological ranges predict (1) a realm in which the complex pattern of Ca^{2+} and cAMP dynamics (**b**) results (red), (2) a domain in which cAMP oscillations are locked to Ca^{2+} oscillations one-to-one (yellow), and (3) a region in which no Ca^{2+} or cAMP oscillations occur (blue).

(Fig. 4d). Selective increase of cAMP by distinct patterns of Ca^{2+} transients appears to result from the match of AC and PDE kinetics with the timescale of Ca^{2+} oscillations. Elimination of any one of the three feedback loops (Fig. 4a) abolishes or dramatically alters the patterns of transients generated by the model (data not shown).

Model simulations make several experimentally testable predictions. In particular, the model predicts that there is an optimal

pattern of Ca^{2+} transients for eliciting increases of cAMP. We determined the cAMP response, defined as the ratio of signal amplitude (μM) to signal duration (s), as a function of intraburst Ca^{2+} spike frequency and Ca^{2+} spike burst duration (Fig. 4e). Contours in the x – y plane indicate combinations of spike number/burst and intraburst frequency yielding equivalent cAMP responses. Because the number of spikes within a burst that elicits the maximum response corresponds to that identified in experimental records, the biological system appears to be tuned to its optimum performance; the triplet maximum arises from the dynamics of activation of AC and PDE. The model also predicts the result of coincident changes in Ca^{2+} and cAMP metabolism. Simulations altering $[\text{InsP}_3]$ and AC activity simultaneously reveal conditions producing distinct linkages of Ca^{2+} and cAMP transients (Fig. 4f). These changes in their dynamical patterns make possible differential activation of kinases and gene expression.

Hormones, growth factors, and drugs elicit cellular responses in the form of Ca^{2+} oscillations, the frequencies of which are determined by the amount and type of the agent^{18–21}. Patterns of spontaneous and stimulated Ca^{2+} transients drive transcription of specific genes^{22–24}, and stimulated cAMP transients also regulate gene expression^{25,26}. We have shown that only certain bursts of Ca^{2+} spikes generate a cAMP increase. Our results may be useful in understanding the coding of gene expression by interactions between Ca^{2+} transients and cAMP oscillations. Higher frequencies of Ca^{2+} spikes have been shown to control gene expression more directly via Ca^{2+} -dependent kinases that are not efficiently activated by low frequencies of Ca^{2+} transients. For example, cAMP increases induced by serotonin at frequencies similar to those reported here lead to translocation of PKA catalytic subunits into the nucleus of *Aplysia* neurons and gene expression underlying long-term facilitation^{25,27,28}. The theoretical model combining Ca^{2+} oscillatory machinery and feedback regulation of cAMP synthesis predicts the existence of these low-frequency cAMP transients rather than high-frequency events²⁹. These results suggest that it could be fruitful to investigate interactions among other classes of second messengers³⁰, as tiers of messenger systems may be necessary to generate different patterns of transients that produce unique cellular responses to stimulation. □

Methods

Cell culture

Spinal neurons from neural plate stage *Xenopus* embryos were cultured³ and allowed to develop for 8–12 h (young neurons) or 18–24 h (mature neurons). During imaging experiments, cells were usually superfused at a rate of 5 ml min^{-1} with saline containing 2 mM Ca^{2+} . Ca^{2+} spikes were blocked by substitution with a Ca^{2+} -free medium containing 1 mM EGTA or by addition of a mixture containing $1 \mu\text{M}$ GVIA ω -conotoxin (Alomone), $0.02 \mu\text{M}$ calcludine (Calbiochem), $1 \mu\text{M}$ flunarizine and $1 \mu\text{g ml}^{-1}$ tetrodotoxin (Sigma). Ca^{2+} spikes were stimulated by 5-s pulses of saline containing 100 mM KCl (ref. 3).

Imaging

Intracellular Ca^{2+} was measured using Fluo-4 AM (Molecular Probes). Cells were incubated in $5 \mu\text{M}$ indicator for 40 min and washed. Intracellular cAMP was measured using FICHR (Molecular Probes; average stock concentration $\sim 20 \mu\text{M}$) injected into single cell somas in 1 mM Ca^{2+} medium with a microinjector system (Eppendorf). Injection pressure was $\sim 170 \text{ hPa}$ and capillary holding pressure was $\sim 130 \text{ hPa}$. Neurons were imaged with a BioRad MRC-1024 confocal imaging system with a 15-mW Kr/Ar ion laser and an Olympus microscope with 60 $\times$ objective (NA 0.9). At 1–3% of full power, the laser provided excitation at 488 nm wavelength, suitable for both dyes. The Fluo-4 emission filter had a bandpass of 535/22 nm and those of the FICHR emission filters were 540/30 nm and 580/32 nm. For cAMP measurements two single-wavelength emission images were simultaneously acquired every 10 s; changes in fluorescence were observed for $>20 \text{ min}$ in response to sustained application of $50 \mu\text{M}$ forskolin. For Ca^{2+} imaging the rate of acquisition was 0.2 Hz .

Data analysis

Fluo-4 fluorescence was analysed with NIH Image (W. Rasband). Changes were assessed by average pixel intensity within cell bodies. Only fluorescence increases above 150% of baseline were scored as spikes. For analysis of FICHR fluorescence, traces were corrected for photobleaching, background was subtracted and the ratio of 520/580 nm emission was calculated. At low laser power, no autofluorescence was detected. Cells were studied only if

they responded to forskolin stimulation, and cAMP transients were scored only if 520- and 580-nm traces changed in opposite directions. Statistical analysis was performed using the Kolmogorov–Smirnov non-parametric test; values are presented as mean $\pm$ s.e.m. and were considered significantly different for $P < 0.05$.

Computation

Constants in the model are annotated in Supplementary Information; MATLAB 6.0 (Mathworks) was used for numerical calculations. Wavelet analysis was performed using the Morlet wavelet from the standard numerical 'wavelet analysis' package (Mathworks). Wavelet analysis produces a set of coefficients, C_{ab} calculated using the formula $C_{ab} \approx \int dt f(t) \Psi[(t - a)/b]$, where Ψ is the analysing function referred to as the 'wavelet', a represents time localization and b is the scale, which is inversely proportional to the local frequency. To avoid edge effects, only the central 40 min of each hour were considered. No periodic structure was evident when this method was tested with white noise.

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- Cuthbertson, K. S. & Cobbold, P. H. Phorbol ester and sperm activate mouse oocytes by inducing sustained oscillations in cell Ca^{2+} . *Nature* **316**, 541–542 (1985).
- Kline, D. & Kline, J. T. Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. *Dev. Biol.* **149**, 80–89 (1992).
- Gu, X. & Spitzer, N. C. Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca^{2+} transients. *Nature* **375**, 784–787 (1995).
- Fields, R. D., Eshete, E., Stevens, B. & Itoh, K. Action potential-dependent regulation of gene expression: temporal specificity in Ca^{2+} -cAMP-responsive element binding proteins, and mitogen-activated protein kinase signalling. *J. Neurosci.* **17**, 7252–7266 (1997).
- Carey, M. B. & Matsumoto, S. G. Spontaneous calcium transients are required for neuronal differentiation of murine neural crest. *Dev. Biol.* **215**, 298–313 (1999).
- Wayman, G. A., Hinds, T. R. & Storm, D. R. Hormone stimulation of type III adenylyl cyclase induces Ca^{2+} oscillations in HEK-293 cells. *J. Biol. Chem.* **270**, 24108–24115 (1995).
- Rasmussen, H. *Calcium and cAMP as Synaptic Messengers* (Wiley, New York, 1981).
- Ming, G. L. *et al.* cAMP-dependent growth cone guidance by netrin-1. *Neuron* **19**, 1225–1235 (1997).
- Mons, N., Decorte, L., Jaffard, R. & Cooper, D. M. F. Ca^{2+} -sensitive adenylyl cyclases, key integrators of cellular signalling. *Life Sci.* **62**, 1647–1652 (1998).
- Bhalla, U. S. & Iyengar, R. Emergent properties of networks of biological signalling pathways. *Science* **283**, 381–387 (1999).
- Hartwell, L. H., Hopfield, J. J., Leibler, S. & Murray, A. W. From molecular to modular cell biology. *Nature* **402** suppl., C47–C52 (1999).
- Tang, Y. & Othmer, H. G. Frequency encoding in excitable systems with applications to calcium oscillations. *Proc. Natl Acad. Sci. USA* **92**, 7869–7873 (1995).
- Islam, M. S. *et al.* *In situ* activation of the type 2 ryanodine receptor in pancreatic beta cells requires cAMP-dependent phosphorylation. *Proc. Natl Acad. Sci. USA* **95**, 6145–6150 (1998).
- Haug, L. S., Jensen, V., Hvalby, O., Walaas, S. I. & Ostvold, A. C. Phosphorylation of the inositol 1,4,5-trisphosphate receptor by cyclic nucleotide-dependent kinases *in vitro* and in rat cerebellar slices *in situ*. *J. Biol. Chem.* **274**, 7467–7473 (1999).
- Eliot, L. S., Dudai, Y., Kandel, E. R. & Abrams, T. W. Ca^{2+} /calmodulin sensitivity may be common to all forms of neural adenylyl cyclase. *Proc. Natl Acad. Sci. USA* **86**, 9564–9568 (1989).
- Sette, C., Vicini, E. & Conti, M. The rat PDE3/IVD phosphodiesterase gene codes for multiple proteins differentially activated by cAMP-dependent protein kinase. *J. Biol. Chem.* **269**, 18271–18274 (1994).
- Beck, F., Blasius, B., Lutttge, U., Neff, R. & Rascher, U. Stochastic noise interferes coherently with a model biological clock and produces specific dynamic behaviour. *Proc. R. Soc. Lond. B* **268**, 1307–1313 (2001).
- Woods, N. M., Cuthbertson, K. S. & Cobbold, P. H. Repetitive transient rises in cytoplasmic free calcium in hormone-stimulated hepatocytes. *Nature* **319**, 600–602 (1986).
- Berridge, M. J. & Galione, A. Cytosolic calcium oscillators. *FASEB J.* **2**, 3074–3082 (1988).
- Fewtrell, C. Ca^{2+} oscillations in non-excitable cells. *Annu. Rev. Physiol.* **55**, 427–454 (1993).
- Goldbeter, A., Dupont, G. & Halloy, J. The frequency encoding of pulsatility. *Novartis Found. Symp.* **227**, 19–36 (2000).
- Haisenleder, D. J., Yasin, M. & Marshall, J. C. Gonadotropin subunit and gonadotropin-releasing hormone receptor gene expression are regulated by alterations in the frequency of calcium pulsatile signals. *Endocrinology* **138**, 5227–5230 (1997).
- Villalobos, C., Faught, W. J. & Frawley, L. S. Dynamic changes in spontaneous intracellular free calcium oscillations and their relationship to prolactin gene expression in single, primary mammatropes. *Mol. Endocrinol.* **12**, 87–95 (1998).
- Dolmetsch, R. E., Xu, K. L. & Lewis, R. S. Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* **392**, 933–936 (1998).
- Kaang, B. K., Kandel, E. R. & Grant, S. G. Activation of cAMP-responsive genes by stimuli that produce long-term facilitation in *Aplysia* sensory neurons. *Neuron* **10**, 427–435 (1993).
- Haisenleder, D. J., Yasin, M. & Marshall, J. C. Enhanced effectiveness of pulsatile 3' 5' cyclic adenosine monophosphate in stimulating prolactin and alpha-subunit expression. *Endocrinology* **131**, 3027–3033 (1992).
- Bacskai, B. J. *et al.* Spatially resolved dynamics of cAMP and protein kinase A subunits in *Aplysia* sensory neurons. *Science* **260**, 222–226 (1993).
- Montarolo, P. G. *et al.* A critical period for macromolecular synthesis in long-term heterosynaptic facilitation in *Aplysia*. *Science* **234**, 1249–1254 (1986).
- Cooper, D. M. F., Mons, N. & Karpen, J. W. Adenylyl cyclases and the interaction between calcium and cAMP signalling. *Nature* **374**, 421–424 (1995).
- Berridge, M. J., Lipp, P. & Bootman, M. D. The versatility and universality of calcium signalling. *Nature Rev. Mol. Cell. Biol.* **1**, 11–21 (2000).

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Competing interests statement

The authors declare that they have no competing financial interests.

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Determination of left–right patterning of the mouse embryo by artificial nodal flow

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Substantial insight has recently been achieved into the mechanisms responsible for the generation of left–right (L–R) asymmetry in the vertebrate body plan^{1–4}. However, the mechanism that underlies the initial breaking of symmetry has remained unclear. In the mouse, a leftward fluid flow on the ventral side of the node caused by the vortical motion of cilia (referred to as nodal flow) is implicated in symmetry breaking⁵, but direct evidence for the role of this flow has been lacking. Here we describe the development of a system in which mouse embryos are cultured under an artificial fluid flow and with which we have examined how flow affects L–R patterning. An artificial rightward flow that was sufficiently rapid to reverse the intrinsic leftward nodal flow resulted in reversal of situs in wild-type embryos. The artificial flow was also able to direct the situs of mutant mouse embryos with immotile cilia. These results provide the first direct evidence for the role of mechanical fluid flow in L–R patterning.

The breaking of symmetry during vertebrate development is thought to occur in or near the node. In the mouse embryo, the ventral surface of the node (nodal pit) possesses several hundred monocilia⁶ that rotate in a clockwise direction, and this rotational movement somehow generates a leftward laminar flow⁵. The lack of this nodal flow as a result of impaired ciliary development leads to randomization of body situs^{5,7–10}. Nodal flow has thus been proposed to trigger a signalling cascade responsible for L–R patterning by transporting an unidentified molecule towards the left side of the body. However, this 'nodal flow hypothesis' has remained unproved. It raises many questions, the most important of which is whether the nodal flow is a genuine symmetry-breaking event or simply a manifestation of such an event.

One approach to test directly the role of nodal flow would be to manipulate the flow mechanically and to examine how such manipulation affects L–R patterning. To achieve this goal, we developed a culture system (flow culture) in which embryos are allowed to develop under a constant flow of culture medium driven by a peristaltic pump (Fig. 1a). In this system, octopus-trap-like pots hold embryos in their proximal region (Fig. 1b, c), so that the distal region, including the node, is exposed to the flow. The artificial flow can be controlled in two ways: First, the flow can be leftward (Fig. 1b) or rightward (Fig. 1c) with respect to the L–R axis of the embryo, depending on how the embryos are placed in the pots. Second, the rate of the flow can be adjusted to either fast or slow.

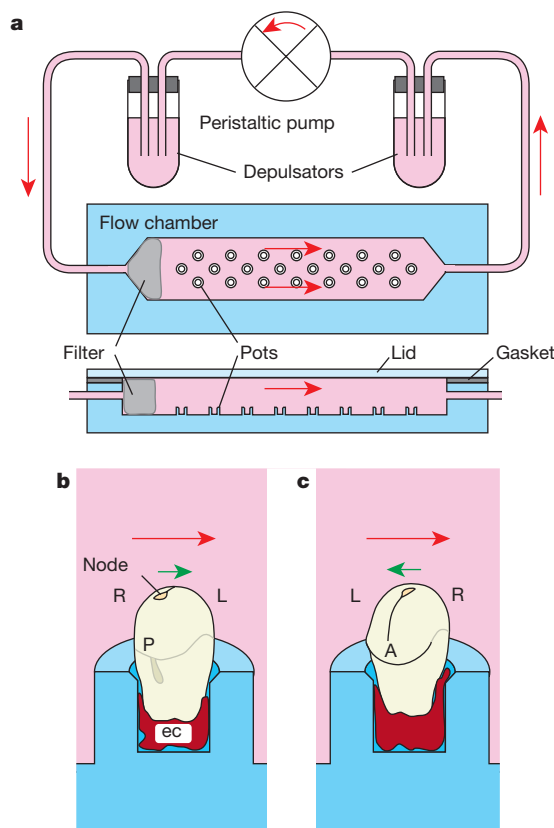


Figure 1 Flow culture system. **a**, Schematic representation of the flow culture system. The peristaltic pump and depulsators ensure constant circulation of culture medium. The filter located at the entrance of the flow chamber prevents turbulence in the chamber. **b, c**, Relation between the direction of the intrinsic nodal flow (green arrow) and that of the pump-driven artificial flow (red arrow). Artificial flow is leftward in **b** and rightward in **c**, with respect to the L–R axis of each embryo. The ectoplacental cone (ec) serves as a glue to stabilize the position of the embryo. A, P, L and R refer to the anterior, posterior, left and right sides of the embryo, respectively.

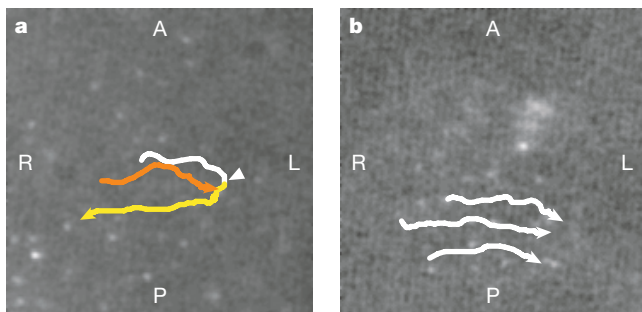


Figure 2 Reversal of the intrinsic nodal flow by fast, but not by slow, rightward artificial flow. Embryos at the one-somite stage were placed in the flow chamber as depicted in Fig. 1c, and the fluid flow in the node was visualized with fluorescent beads. Arrows indicate the direction of individual beads. Video clips of bead motion are provided in the Supplementary Information. **a**, Fast rightward flow. The pump was initially off, then it was turned on to impose the fast rightward flow, and it was then turned off again. In the absence of the artificial flow, a bead moved leftward (white line), but it turned rightward (yellow line) when the pump started (arrowhead). After the pump stopped, a bead resumed its original leftward movement (orange line). The bead travelled along the L–R axis at the speed of $12.9 \mu\text{m s}^{-1}$ (white line), $14.5 \mu\text{m s}^{-1}$ (yellow line), and $16.3 \mu\text{m s}^{-1}$ (orange line). **b**, Slow rightward flow. The flow in the node was observed under the slow rightward flow. The flow remained leftward even when the slow rightward flow was imposed (white lines). Three beads from the top travelled along the L–R axis at the speed of $19.4 \mu\text{m s}^{-1}$, $16.1 \mu\text{m s}^{-1}$ and $22.4 \mu\text{m s}^{-1}$, respectively. A, P, L and R refer to anterior, posterior, left and right sides of the node, respectively.

According to hydrodynamics, the rate of fluid flow decreases in the region near the surface of an object. This principle results in the formation of a velocity gradient of the pump-driven flow in the vicinity of each embryo, with the flow rate decreasing as the distance to the embryo shortens. Given that nodal flow occurs only within a distance of several micrometres from the surface of the node, the pump-driven flow in this region would be expected to exert limited influence compared with that predicted from the average flow rate in the chamber. It was therefore essential to examine how endogenous nodal flow was affected by the pump-driven flow. Although we tested several different speeds, we describe the effects of two rates of pump-driven flow in the following experiments: fast flow (average speed in the chamber of $110 \mu\text{m s}^{-1}$) and slow flow (average speed in the chamber of $5.7 \mu\text{m s}^{-1}$). Mouse embryos were cultured under conditions of fast flow or slow flow, and the fluid flow within the node was visualized with fluorescent beads. Fast rightward flow in the chamber was sufficient to reverse the intrinsic nodal flow of the normal mouse embryo (Fig. 2a; Supplementary Information 1), whereas slow rightward flow was not (Fig. 2b; Supplementary Information 2). Leftward flow in the chamber, either fast or slow, did not change the direction of the fluid flow in the node (data not shown).

We next tested four types of artificial flow (leftward or rightward, fast or slow) for their effects on L–R patterning. Mouse embryos at the presomite stage were cultured in the flow chamber for 14 h, followed by conventional rotation culture¹¹ for an additional 32 h. At the end of the incubation, the directions of heart looping and embryonic turning were examined. The *Pitx2-lacZ* transgene (*17-P1*)¹² was also used as a marker for L–R patterning, and embryos harbouring this construct were stained with the β -galactosidase substrate X-gal. Under conditions of fast leftward or slow leftward flow, most wild-type embryos developed a normal L–R axis (Fig. 3a, b, h). They thus manifested dextral heart looping (D-looping) and normal embryonic turning. The *Pitx2-lacZ* transgene exhibited normal left-sided expression in the common atrium chamber, and left-sided or bilateral expression in the truncus arteriosus. When the embryos treated with fast leftward flow were recovered earlier (after 10 h of rotation culture instead of 32 h), *Pitx2-lacZ* showed left-sided expression in the lateral plate (Fig. 3i). In contrast, fast rightward flow efficiently reversed the L–R markers of wild-type embryos (Fig. 3d, j, k, l). Thus, most of the embryos exhibited reversed heart looping (L-looping), reversed turning, and right-sided expression of *Pitx2-lacZ* and *nodal*. Slow rightward flow, which was not fast enough to reverse the direction of the endogenous nodal flow (Fig. 2b), failed to induce frequent reversal of L–R situs (Fig. 3c). The effects of fast rightward flow were dependent on embryo stage. Whereas embryos at the presomite stage were sensitive to fast rightward flow, those at the one-somite (Fig. 3e), two-somite (Fig. 3f), or three-somite (Fig. 3g) stage were not.

We performed similar experiments with homozygous *inversus viscerum* (*iv/iv*) mutant embryos, which both lack nodal flow because the monocilia in the node are immotile^{7,9} and exhibit randomized L–R patterning (half of *iv/iv* homozygotes thus develop situs inversus). If the lack of nodal flow is responsible for the observed L–R defects in *iv/iv* embryos, then an artificial flow that mimics the nodal flow might be expected to rescue the phenotype. Indeed, *iv/iv* embryos cultured at the presomite stage under conditions of fast leftward or slow leftward flow manifested normal turning, D-looping of the heart tube, and left-sided expression of the *Pitx2-lacZ* transgene (Fig. 4a, b). In contrast, most *iv/iv* embryos subjected to fast rightward or slow rightward flow exhibited reversal of L–R situs (Fig. 4c, d). Although slow rightward flow was unable to change the direction of the endogenous nodal flow of wild-type embryos (Fig. 2b), slow flow was able to induce L–R patterning in *iv/iv* embryos with immotile cilia.

The changes in L–R patterning induced by artificial fluid flow are unlikely to be due to nonspecific effects of the *in vitro* culture. First, embryos subjected to rightward and leftward flows were cultured

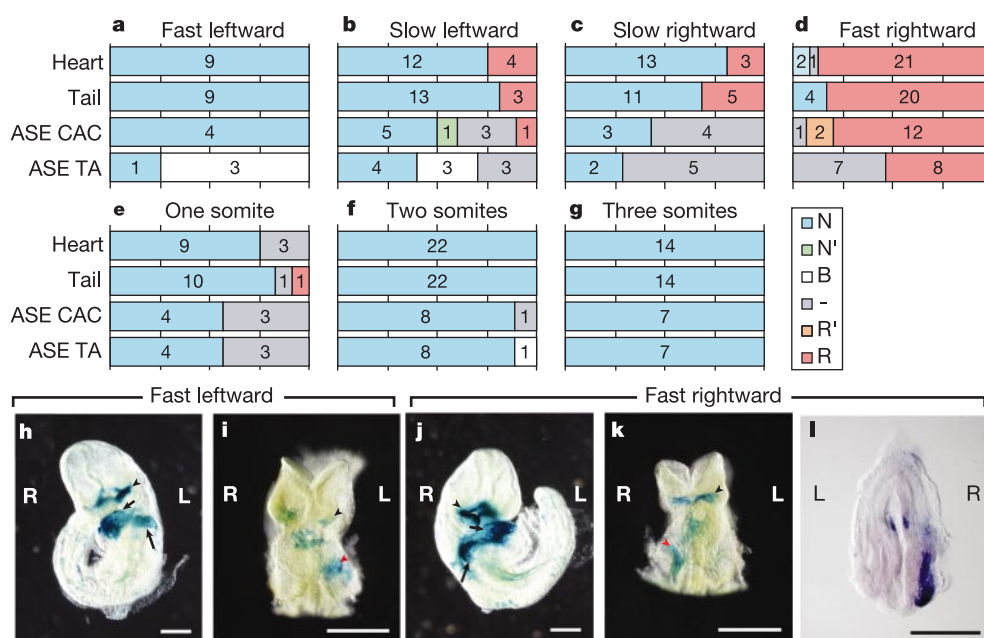


Figure 3 Reversal of situs of wild-type embryos at the presomite stage by fast rightward flow. The effects of artificial fluid flow on various L–R markers were evaluated. **a–d**, Mouse embryos at the presomite stage were cultured under the indicated conditions of artificial flow. Most embryos subjected to fast (**a**) or slow (**b**) leftward flow or to slow rightward flow (**c**) developed normal situs. In contrast, fast rightward flow (**d**) resulted in reversal of situs in most embryos. **e–g**, Embryos at the one-somite (**e**), two-somite (**f**), or three-somite (**g**) stage were insensitive to fast rightward flow. Colour scale: N, normal patterns (D-looping for the heart, right-sided tail for embryonic turning, and left-sided expression for *Pitx2-lacZ*; N', bilateral expression of *Pitx2-lacZ*, with a higher level of expression on the left side; B, bilaterally equal expression of *Pitx2-lacZ*; (–), absence of L–R markers (outloop in the heart, no embryonic turning, and no *Pitx2-lacZ* expression); R', bilateral expression of *Pitx2-lacZ*, with a higher level of expression on the right side; R, inverted patterns (L-looping for the heart, left-sided tail for embryonic turning, and right-sided expression for *Pitx2-lacZ*). CAC, common atrium chamber; TA, truncus arteriosus; ASE, transgene expression determined by the asymmetric enhancer of *Pitx2*. Numbers refer to the number of embryos with the indicated properties among the total examined. **h**,

Presomite-stage embryo subjected to fast leftward flow and then stained with X-gal. D-looping of the heart, normal turning, left-sided X-gal staining in the CAC (long arrow), and bilateral X-gal staining in the TA (short arrow) are apparent. Bilateral staining in the first branchial arch (arrowhead) is also characteristic of this transgene¹². **i**, Presomite-embryo treated with fast leftward flow was recovered earlier (after 10 h of rotation culture instead of the typical 32 h) and was stained with X-gal. Left-sided staining in the lateral plate (red arrowhead) and bilateral staining in the branchial arch (black arrowhead) are apparent. **j**, Presomite-stage embryo subjected to fast rightward flow and then stained with X-gal. L-looping of the heart, reversed turning, right-sided X-gal staining in the CAC (long arrow) and TA (short arrow) are apparent. The first branchial arch is indicated with a black arrowhead. **k**, Presomite-embryo treated with fast rightward flow was recovered earlier (after 10 h of rotation culture instead of the typical 32 h) and was stained with X-gal. Right-sided staining is apparent in the lateral plate (red arrowhead). The first branchial arch is indicated with a black arrowhead. **l**, Presomite-embryos treated with fast rightward flow were recovered earlier and were examined for *nodal* expression by *in situ* hybridization. *nodal* expression in the lateral plate was right-sided. All scale bars, 0.5 mm.

simultaneously in the same chamber (Fig. 1b, c), but the effects on L–R patterning were specific to the direction of the artificial flow with respect to the L–R axis of each embryo. Second, whereas the slow rightward flow failed to affect the situs of wild-type embryos, it induced reversal of situs in *iv/iv* embryos. Furthermore, whereas embryos at the presomite stage were sensitive to the fast rightward flow, those at slightly later stages of development were not, suggesting that L–R determination is still undetermined at the presomite stage. This notion is consistent both with the previous observation that nodal flow begins at the presomite stage (the late neural-fold stage)⁷, before asymmetric expression of *nodal* and *lefty* has begun^{13–16}, as well as with the previous suggestion that the L–R axis of the rat embryo is determined at the equivalent stage¹⁷.

Our results provide the first direct evidence for the role of mechanical fluid flow in L–R determination. In the flow culture system, the surface of the whole embryos was subjected to the current of media. However, it is most probably the alteration of flow in the node that resulted in situs reversal because the node is the only region of the embryo surface where the intrinsic directional flow has been detected. Of the questions that remain unanswered, one of the most important concerns the mechanism by which the vectorial flow is generated by the rotational movement of the cilia. Hydrodynamic considerations may provide some insight: it will be essential to characterize precisely the movement and morphology of the cilia, the shape of the node and the hydrodynamic properties of the nodal flow. □

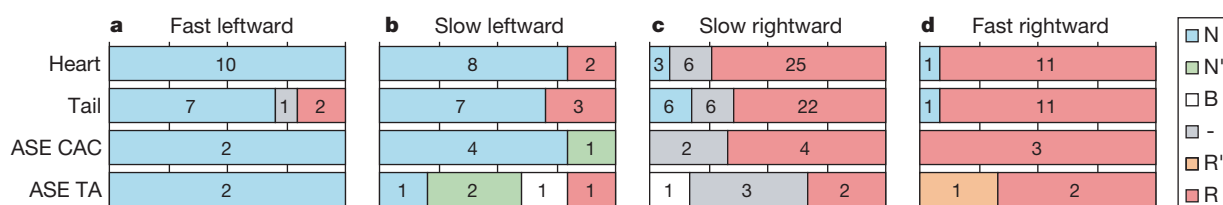


Figure 4 Determination of situs of *iv/iv* embryos by artificial fluid flow. Presomite-stage *iv/iv* embryos were incubated under the indicated conditions of artificial flow, the effects of which were then evaluated as described in Fig. 3. Fast (**a**) and slow (**b**) leftward flows

mostly gave rise to normal situs, whereas slow (**c**) and fast (**d**) rightward flows induced reversal of situs. Colour scale as in Fig. 3.

Methods

Flow culture

Male mice harbouring a *Pitx2-lacZ* transgene (17-P1, a transgene that recapitulates the asymmetric expression of *Pitx2*)¹² were crossed with ICR female mice (Charles River). The resulting embryos were dissected at embryonic day 7.7 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 25 mM HEPES-NaOH (pH 7.2). Embryos were staged on the basis of their morphology¹⁸, and those between the late-bud and the late-headfold stages were used for flow culture. They were allowed to develop in the flow chamber for 14 h (embryos develop to the four- to seven-somite stage during flow culture; underdeveloped embryos with fewer than four somites were occasionally observed and were discarded without analysis) and were then subjected to conventional rotation culture¹¹ for an additional 32 h. During this treatment, embryos develop to the stage equivalent to embryonic day 9.5. They were then fixed and stained with X-gal by the standard protocol¹¹. The presence of the transgene was determined by X-gal staining at the first branchial arch and by polymerase chain reaction (PCR)-based genotyping. Throughout culture, embryos were maintained at 37 °C under 5% CO₂ and 20% O₂ in standard embryo culture medium composed of 50% rat serum, 50% DMEM buffered with 44 mM NaHCO₃ (pH 7.2), penicillin (50 U ml⁻¹), and streptomycin (50 mg ml⁻¹).

Visualization of fluid flow in the node

To visualize fluid flow in the node, we added a 2% solution of 1-μm-diameter red fluorescent polystyrene beads (FluoSphere F-8821, Molecular Probes) at a volume proportion of 1/500 to the culture medium (50% rat serum, 50% DMEM buffered with 25 mM HEPES-NaOH (pH 7.2)). The fluid flow was visualized with the fluorescent beads that were present at a level of approximately 5 μm higher than the surface of the node where the natural nodal flow is most apparent. Fluorescent images were obtained with a monochrome CCD (charge-coupled device) camera (HAS-2000EX, DITECT) connected to a personal computer, and they were processed with Scion Image software.

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- Beddington, R. S. & Robertson, E. J. Axis development and early asymmetry in mammals. *Cell* **96**, 195–209 (1999).
- Capdevila, J., Vogan, K. J., Tabin, C. J. & Izpisua Belmonte, J. C. Mechanisms of left-right determination in vertebrates. *Cell* **101**, 9–21 (2000).
- Wright, C. V. Mechanisms of left-right asymmetry: What's right and what's left? *Dev. Cell* **1**, 179–186 (2001).
- Hamada, H., Meno, C., Watanabe, D. & Saijoh, Y. Establishment of vertebrate left-right asymmetry. *Nature Rev. Genet.* **3**, 103–113 (2002).
- Nonaka, S. *et al.* Randomization of left-right asymmetry due to loss of nodal cilia generating leftward flow of extraembryonic fluid in mice lacking KIF3B motor protein. *Cell* **95**, 829–837 (1998).
- Sulik, K. *et al.* Morphogenesis of the murine node and notochordal plate. *Dev. Dyn.* **201**, 260–278 (1994).
- Okada, Y. *et al.* Abnormal nodal flow precedes situs inversus in *iv* and *inv* mice. *Mol. Cell* **4**, 459–468 (1999).
- Takeda, S. *et al.* Left-right asymmetry and kinesin superfamily protein KIF3A: new insights in determination of laterality and mesoderm induction by *kif3A*^{-/-} mice analysis. *J. Cell Biol.* **145**, 825–836 (1999).
- Supp, D. M. *et al.* Targeted deletion of the ATP binding domain of left-right dynein confirms its role in specifying development of left-right asymmetries. *Development* **126**, 5495–5504 (1999).
- Pazour, G. J. *et al.* Chlamydomonas IFT88 and its mouse homologue, polycystic kidney disease gene *pkc373*, are required for assembly of cilia and flagella. *J. Cell Biol.* **151**, 709–718 (2000).
- Hogan, B. L. *et al.* *Manipulating The Mouse Embryo: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1994).
- Shiratori, H. *et al.* Two-step regulation of left-right asymmetric expression of *Pitx2*: Initiation by Nodal signalling and maintenance by *Nkx2*. *Mol. Cell* **7**, 137–149 (2001).
- Collignon, J., Varlet, I. & Robertson, E. J. Relationship between asymmetric nodal expression and the direction of embryonic turning. *Nature* **381**, 155–158 (1996).
- Lowe, L. A. *et al.* Conserved left-right asymmetry of nodal expression and alterations in murine situs inversus. *Nature* **381**, 158–161 (1996).
- Meno, C. *et al.* Left-right asymmetric expression of the TGFβ-family member *lefty* in mouse embryos. *Nature* **381**, 151–155 (1996).
- Meno, C. *et al.* Two closely-related left-right asymmetrically expressed genes, *lefty-1* and *lefty-2*: their distinct expression domains, chromosomal linkage and direct neuralizing activity in *Xenopus* embryos. *Genes Cells* **2**, 513–524 (1997).
- Fujinaga, M. & Baden, J. M. Critical period of rat development when sidedness of asymmetric body structures is determined. *Teratology* **44**, 453–462 (1991).
- Downs, K. M. & Davies, T. Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. *Development* **118**, 1255–1266 (1993).

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Competing interests statement

The authors declare that they have no competing financial interests.

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AID mutates *E. coli* suggesting a DNA deamination mechanism for antibody diversification

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After gene rearrangement, immunoglobulin variable genes are diversified by somatic hypermutation or gene conversion, whereas the constant region is altered by class-switch recombination. All three processes depend on activation-induced cytidine deaminase (AID)^{1–7}, a B-cell-specific protein that has been proposed (because of sequence homology¹) to function by RNA editing. But indications that the three gene diversification processes might be initiated by a common type of DNA lesion^{8–11}, together with the proposal that there is a first phase of hypermutation that targets dC/dG¹², suggested to us that AID may function directly at dC/dG pairs. Here we show that expression of AID in *Escherichia coli* gives a mutator phenotype that yields nucleotide transitions at dC/dG in a context-dependent manner. Mutation triggered by AID is enhanced by a deficiency of uracil-DNA glycosylase, which indicates that AID functions by deaminating dC residues in DNA. We propose that diversification of functional immunoglobulin genes is triggered by AID-mediated deamination of dC residues in the immunoglobulin locus with the outcome—that is, hypermutation phases 1 and 2, gene conversion or switch recombination—dependent on the way in which the initiating dU/dG lesion is resolved.

Although AID is required for all three programmes of diversification of rearranged immunoglobulin genes^{2–7}, its mode of action is unknown. The suggestion that AID might act by editing RNA derives from the sequence similarity between AID and Apobec-1 (the catalytic component of the complex that deaminates cytidine 6666 to uracil in the apolipoprotein B messenger RNA, thereby generating a premature stop codon)¹. But whereas the physiological role of Apobec-1 is to deaminate RNA, the function of AID is to potentiate changes in immunoglobulin gene DNA. It has been suggested that all three programmes of diversification might be initiated by a common type of DNA lesion^{8–11}, and also that there is a first phase of hypermutation that is specifically targeted to dC/dG pairs^{12,13}. These considerations led us to propose the model of immunoglobulin gene diversification shown in Fig. 1.

We visualize diversification as being initiated by AID-mediated deamination of cytidine residues in the immunoglobulin loci. Conventionally this would trigger base-excision repair¹⁴, with uracil being removed by uracil-DNA glycosylase (UDG), followed by cleavage at the abasic site by an apyrimidic endonuclease and reinsertion of a dC residue by a DNA polymerase/deoxyribosephosphodiesterase. If instead of being repaired the DNA strand with the dU residue were used as a template for DNA synthesis, then the outcome would be a dC → dT (and a dG → dA) transition (Fig. 1; phase 1A hypermutation). Alternatively, if DNA synthesis were to occur over the abasic site, then both transitions and transversions

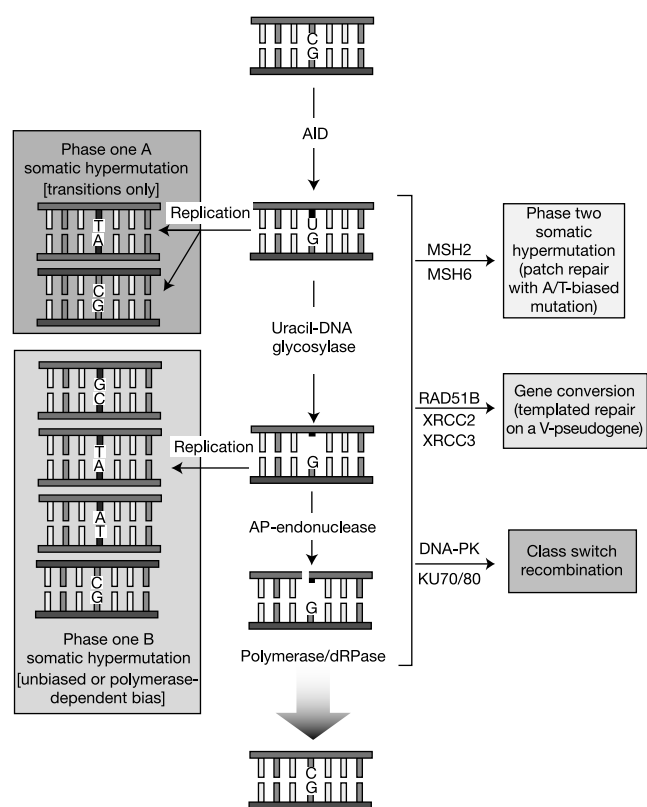


Figure 1 DNA deamination model of immunoglobulin gene diversification. For details, see text. KU70/80 are non-homologous end-joining proteins. AP endonuclease, apyrimidic endonuclease; DNA-PK, DNA-dependent protein kinase; dRPase, deoxyribosephosphodiesterase; V, variable.

would be generated, although a transition bias might still be observed if the polymerase used for the lesion bypass preferentially inserted a dA residue opposite abasic sites (Fig. 1; phase 1B hypermutation). Thus, the stage at which polymerase bypass of the original lesion occurred, as well as the preferences of the polymerase used, would affect the transition bias of the hypermutation. This could account for the observation that mutations in mouse and human and in the hypermutating Ramos B cell line show

a marked transition preference¹⁵, but no such preference is evident in the mutations shown by the XRCC2-deficient chicken DT40 B-cell line¹⁰.

We propose further that template-mediated repair of the deamination-induced lesion by a variable pseudogene would lead to gene conversion; such repair would be dependent on the RAD51 paralogues XRCC2, XRCC3 and RAD51B¹⁰. The second phase of mutation (yielding mutations at dA/dT) that is observed *in vivo* in human and mouse would be triggered by MSH2/MSH6 recognition of the dU/dG mismatch itself or of an intermediate in its correction^{12,13}, and would presumably occur by a form of patch repair. This might involve polymerase η ^{16,17}. Repair partnered on another switch region could lead to switch recombination. For switching, in which non-homologous end-joining has been implicated^{18,19}, one might imagine that deamination of proximal dCs on opposite strands could generate the previously proposed staggered DNA breaks²⁰.

A central component of this model is that AID has the ability to trigger dC $\rightarrow$ dU deamination in DNA. Such an activity would presumably be restricted mostly to its physiological target (the immunoglobulin loci), because a rampant DNA deaminase activity would probably be harmful to the cell. As the activity might be only weak, we determined whether expression of AID could be mutagenic in *E. coli*, because a bacterial read-out would provide a sensitive assay.

A plasmid containing a human AID complementary DNA expressed under the control of a *trp/lac* (*tac*) hybrid promoter was transformed into *E. coli* strain KL16, and its effect on the frequency of mutation to rifampicin resistance (*Rif*^R) was measured by fluctuation analysis (Fig. 2 and Table 1). Mutation to *Rif*^R occurs normally and at low frequency by base substitutions in the *rpoB* gene²¹. In several experiments in the presence of the transcriptional inducer isopropylthiogalactoside (IPTG), the AID-transformed cells generated *Rif*^R colonies at a median frequency that was 4–8 times higher than that of vector-transformed controls (mean 5.4 ± 0.9 ; Table 1). This stimulation was evident in different genetic backgrounds (KL16, GM1003 and AB1157), was dependent on AID (monitored with and without IPTG) and was not restricted to the selection applied, being also evident when mutation to nalidixic acid (*Nal*), valine or fucose resistance was monitored (Fig. 2 and Table 1). The variation in the mutation enhancement observed in the different selections might reflect differences in the types and abundances of mutation that allow growth.

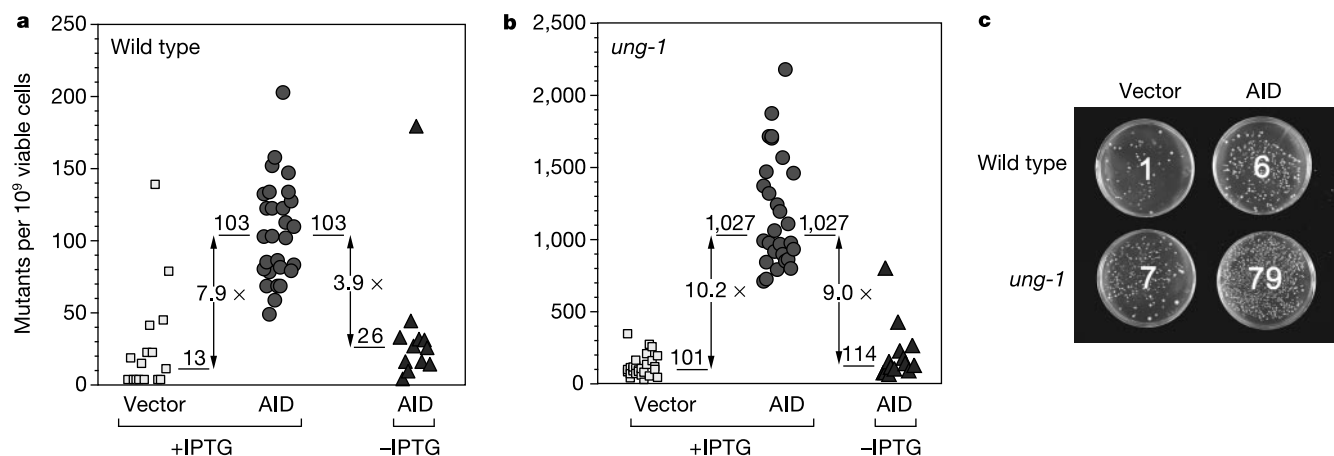


Figure 2 Expression of AID in *E. coli* yields a mutator phenotype that is enhanced by UDG deficiency. **a**, Frequencies of *Rif*^R mutants generated after overnight culture (with (+) or without (–) IPTG) of *E. coli* KL16 carrying either the AID expression plasmid or the vector control. Each point represents the mutation frequency of an independent overnight culture. The fold enhancement by AID expression is indicated. The slight enhancement of

mutation in AID-transformed cells in the absence of IPTG may be due to promoter leakiness. **b**, Mutation frequency of AID- and vector-transformed, UDG-deficient KL16 *ung-1* cells as in **a**, but note the larger scale on the y-axis. **c**, Representative culture plates. The mutation frequency relative to the vector-transformed wild-type control is indicated. See Table 1 for additional data.

Table 1 AID expression stimulates mutation in *Escherichia coli*

Strain	Relevant genotype	Selection	Median mutation frequency ($\times 10^{-9}$) [*]		Fold enhancement by AID
			(-) AID	(+) AID	
KL16	<i>ung</i> ⁺	Rifampicin	14	85	6.1
			11	62	5.6
			41	180	4.4
		Nalidixic acid	1.8	5.2	2.9
			10	35	3.5
		Valine	9.4	76	8.1
	<i>ung-1</i>	Rifampicin	2.0	90	45
			17	31	1.8
			9.6	39	4.1
		Nalidixic acid	100	1,100	11
			78	1,800	23
			110	960	8.7
		Valine	9.7	62	6.4
			29	620	21
		Fucose	5.2	630	120
GM1003	<i>ung-1 mug</i> ⁺	Rifampicin	4.5	310	69
		Rifampicin	42	260	6.2
		Rifampicin	24	240	10
	<i>ung</i> ⁺ <i>mug::mini-Tn10</i>	Rifampicin	29	730	25
		Rifampicin	18	110	6.1
		Rifampicin	23	810	35
AB1157	<i>nfi</i> ⁺	Rifampicin	24	100	4.2
			21	110	5.2
	<i>nfi-1</i>	Rifampicin	36	150	4.2
			40	160	4.0

^{*} Each median mutation frequency was determined from 8–16 independent cultures.

If the AID-mediated enhancement in mutation frequency were due to a stimulation of dC deamination, then the pattern of mutation to Rif^R would show a shift towards dC → dT and dG → dA transitions. The sequence of the *rpoB* gene in several independent Rif^R colonies showed that this is so. Such transitions accounted for 80% of the mutations scored in the AID-transformed cells, but for only 31% of the mutations in the vector-transformed controls (Fig. 3a, b). Given the extent of mutation stimulation by AID, these data are consistent with all of the AID-mediated enhancement being caused by transitions at dC/dG. A similar conclusion was obtained by examining the spectrum of *gyrA* mutations in the Nal^R colonies despite the fact that the selected mutations seemed to be restricted to essentially three nucleotide positions: 34% of the *gyrA* mutations among the control transformants were nucleotide transitions at dC/dG, as compared with 71% in the AID transformants (Fig. 3c).

Notably, there was a difference in mutation distribution between the AID transformants and controls. Analysis of the *rpoB* mutations among the vector-transformed control cells showed that dC → dT or dG → dA transitions at positions Ser 512, Asp 516, Ser 522, His 526, Ser 531, Pro 564 and Ser 574 could all confer Rif^R; however, transitions at only some of these positions (His 526, Ser 531 and Ser 574) were enhanced in the AID transformants and other positions (Asp 516, Ser 522 and Pro 564) showed little sign of increased mutation. More notable was that a common dG → dA transition in the AID transformants at Arg 529 was not seen in any of the controls (Fig. 3a). Similar evidence of specific targeting came from *gyrA*. Whereas dC → dT transitions at Ser 83 and dG → dA transitions at Asp 87 could both confer Nal^R, the dC → dT transitions at Ser 83 were enhanced selectively by AID (Fig. 3c). Despite this strong evidence that AID-dependent mutation was non-random, and presumably dependent on local sequence environment, with these data-sets we could not discriminate whether this sequence preference reflected a hotspot preference similar to that of the dC/dG-biased phase antibody hypermutation¹² because there were only a limited number of base substitutions that could yield the selected phenotypes.

If AID-induced mutations in the *E. coli* transformants were

occurring through deamination of dC, an enhancement of the effect would be expected in cells that lack UDG (coded for by *ung*) because this enzyme has a principal role in the repair of such mutagenic deamination¹⁴. We found this to be the case. Although both UDG deficiency and ectopic expression of AID were sufficient in themselves to yield a mutator phenotype, AID expression in an Ung⁻ background yielded a mutation frequency that was much greater than the sum of their independent mutation frequencies (Fig. 2 and Table 1). In addition, the distribution of mutations in the Ung⁻ background provided strong confirmation that AID mutates by generating transitions at dC/dG pairs, but with the choice of dC/dG pairs targeted being highly non-random (Fig. 4). Given the increased AID-mediated stimulation of mutation in the Ung⁻ background, essentially all the *rpoB* mutations that were detected could be attributed to the action of AID. All 20 mutations analysed were transitions at dC/dG pairs, and of these 19 were found at only two of the seven sites that were capable of generating Rif^R by such transitions (Fig. 4).

In *E. coli ung-1* mutants, some back-up UDG activity may be provided by the product of the *mug* gene²². We found that although the AID mutator effect was not significantly higher in a Mug⁻ than in a Mug⁺ background, the *mug::mini-Tn10* mutation allowed at most a slightly augmented AID mutator effect when combined with *ung-1* (Table 1). If AID were to act by deaminating dG rather than dC, one might anticipate an increased mutation frequency in a background deficient in endonuclease V (coded for by *nfi*) because this enzyme is implicated in the repair of deoxyxanthosine^{23,24}. This did not occur; the mutation frequency shown by AID-transformed *nfi-1* cells approximated the sum of the frequencies that were independently attributable to AID and *nfi-1* (Table 1).

Our data indicate that AID triggered the deamination of dC residues in DNA. It will be interesting to ascertain whether AID is sufficient to mediate dC deamination directly *in vitro* or whether it works with or through other factors. The homology of AID to Apobec-1 and cytidine deaminases¹ obviously argues in favour of a close involvement of AID in the DNA deamination process itself. The preferential targeting of mutation to the immunoglobulin loci in lymphocytes presumably depends on the proteins with which

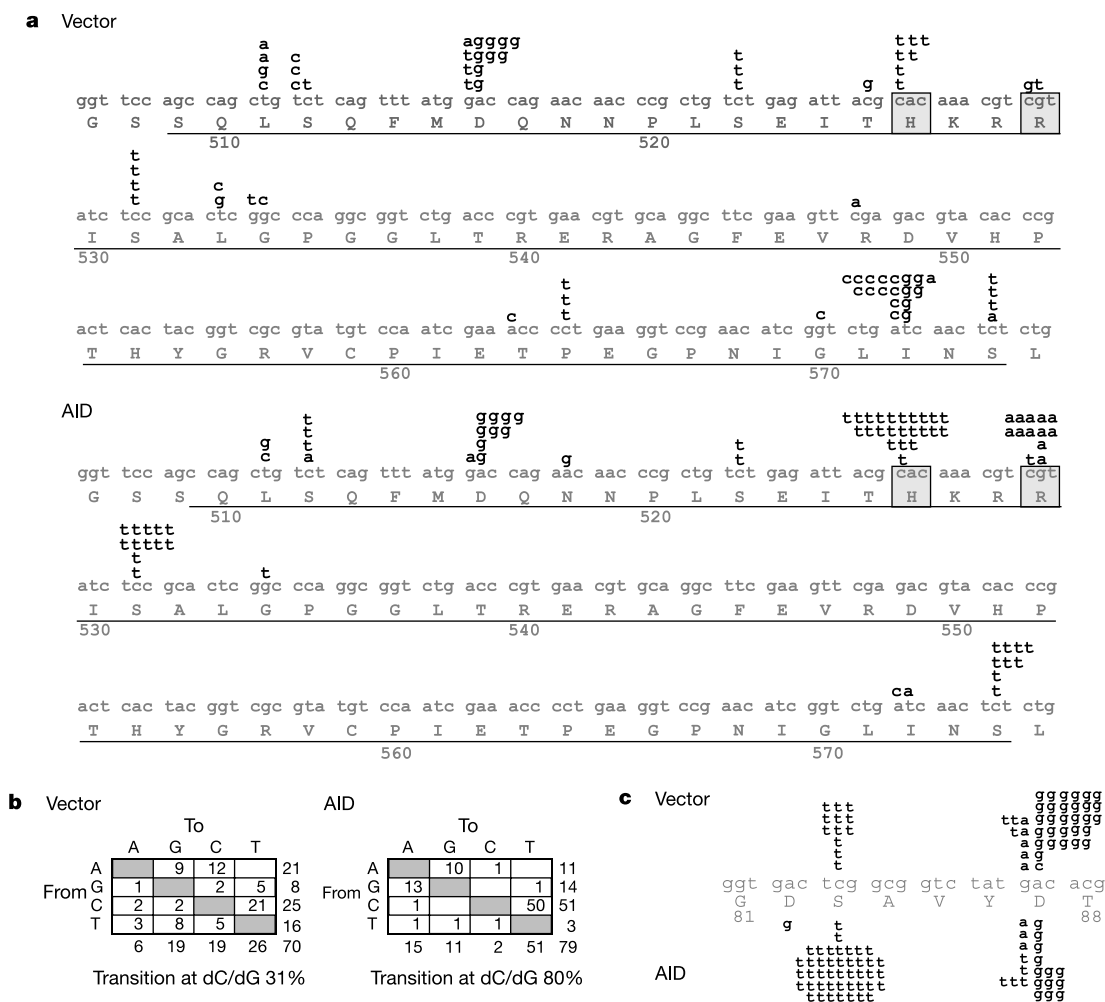


Figure 3 Nature of the AID-induced mutations in an UDG-positive (*ung*⁺) background. **a**, Comparison of the distribution of independent *rpoB* mutations identified in Rif^R colonies obtained from AID- and vector-transformed cells. Data are combined from results obtained using both KL16 and AB1157 hosts, but the two hosts showed no difference in their mutation spectrum. The underlined sequence is the region of *rpoB* that carries most mutations conferring Rif^R (ref. 30). Less than 5% of the Rif^R sequenced clones did not

show any mutations in this region. **b**, Comparison of the pattern of *rpoB* nucleotide substitutions identified in Rif^R colonies obtained from AID- and vector-transformed cells. **c**, Comparison of the independent *gyrA* mutations identified in Nal^R colonies of AID- and vector-transformed *E. coli* KL16. Less than 5% of the Nal^R clones analysed did not show mutations in the sequenced region.

AID associates. Given that the *cis*-regulation of both switch recombination and hypermutation is linked to the transcription regulatory elements^{18,25}, it seems likely that AID is recruited either directly or indirectly by transcription- or chromatin-associated factors.

There are several members of the AID/apobec/phorbolin family in humans²⁶, and it is possible that AID is not the only member that can trigger mutation. Although an ability to deaminate dC in DNA can be shown in bacterial site-specific DNA methylases²⁷, homology considerations²⁸ make it seem likely that AID evolved from a deaminase such as one working on free cytidine. But it may be that DNA deaminating activities go back a long way—repeat-induced point mutation in *Neurospora*²⁹ is characterized by a high frequency of nucleotide transitions at dC/dG pairs, which suggests that phase 1 of antibody hypermutation may have ancient antecedents. □

Methods

Constructs and strains

The AID expression plasmid was generated by cloning the human AID cDNA⁵ in an *NcoI*/*HindIII* fragment into pTrc99A (Pharmacia; gift of R. Savva). *E. coli* strains KL16 (Hfr (PO-45) *relA1 spoT1 thi-1*) and its *ung-1* derivative (BW310), as well as AB1157 and its *nfi-1::cat* derivative (BW1161), were from B. Weiss; GM1003 (*dcm-6 thr-1 hisG4*

leuB6 rpsL ara-14 supE44 lacY1 tonA31 tsx-78 galK2 galE2 xyl-5 thi-1 mtl-1 mug::mini-Tn10) derivatives carrying *ung-1* and/or *mug::mini-Tn10* mutations were from A. Bhagwat.

Monitoring mutation

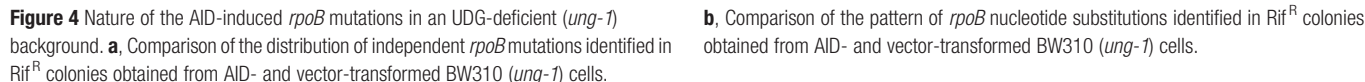
Mutation frequencies were measured by determining the median number of colony-forming cells that survived selection per 10⁹ viable cells plated. Each median in Fig. 2 and Table 1 was determined from 8–16 independent cultures grown overnight to saturation in rich medium supplemented with 100 µg ml⁻¹ carbenicillin and 1 mM IPTG (with the exception of the controls indicated in Fig. 2a, b). IPTG-induced AID expression conferred no obvious defect in cell growth or viability with no effect of AID expression on viable cell counts. Rif^R and Nal^R colonies were selected on rich medium containing 100 µg ml⁻¹ rifampicin and 40 µg ml⁻¹ Nal, respectively. Valine- and fucose-resistant mutants were selected on minimal M9 medium containing 0.2% glucose, 40 µg ml⁻¹ L-valine and 0.1% L-arabinose and 0.2% D-fucose, respectively¹⁹.

Sequence analysis

The nature of the Rif^R and Nal^R mutants was determined by directly amplifying and sequencing the relevant section of *rpoB* (627-base pair PCR product amplified using 5'-TTGGCGAAATGGCGGAAAACC-3' and 5'-CACCACGCGATACACCTGCTG-3') or *gyrA* (521-bp PCR product amplified using oligonucleotides 5'-GCGCGCTGTGTATAATT-3' and 5'-TTCGCTGCCGTCATAGTTATC-3').

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1. Muramatsu, M. *et al.* Specific expression of activation-induced cytidine deaminase (AID), a novel



3. Muramatsu, M. *et al.* Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. *Cell* **102**, 553–563 (2000).
4. Revy, P. *et al.* Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell* **102**, 565–575 (2000).
5. Arakawa, H., Hauschild, J. & Buerstedde, J. M. Requirement of the Activation-Induced Deaminase (AID) gene for immunoglobulin gene conversion. *Science* **295**, 1301–1306 (2002).
6. Harris, R. S., Sale, J. E., Petersen-Mahrt, S. K. & Neuberger, M. S. AID is essential for immunoglobulin V gene conversion in a cultured B cell line. *Curr. Biol.* **12**, 435–438 (2002).
7. Martin, A. *et al.* Activation-induced cytidine deaminase turns on somatic hypermutation in hybridomas. *Nature* **415**, 802–806 (2002).
8. Okazaki, I., Kinoshita, K., Muramatsu, M., Yoshikawa, K. & Honjo, T. The AID enzyme induces class switch recombination in fibroblasts. *Nature* **416**, 340–345 (2002).
9. Maizels, N. Somatic hypermutation: how many mechanisms diversify V region sequences? *Cell* **83**, 9–12 (1995).
10. Weill, J. C. & Reynaud, C. A. Rearrangement/hypermutation/gene conversion: when, where and why? *Immunol. Today* **17**, 92–97 (1996).
11. Sale, J. E., Calandrin, D. M., Takata, M., Takeda, S. & Neuberger, M. S. Ablation of XRCC2/3 transforms immunoglobulin V gene conversion into somatic hypermutation. *Nature* **412**, 921–926 (2001).
12. Ehrenstein, M. R. & Neuberger, M. S. Deficiency in Msh2 affects the efficiency and local sequence specificity of immunoglobulin class-switch recombination: parallels with somatic hypermutation. *EMBO J.* **18**, 3484–3490 (1999).
13. Rada, C., Ehrenstein, M. R., Neuberger, M. S. & Milstein, C. Hot spot focusing of somatic hypermutation in MSH2-deficient mice suggests two stages of mutational targeting. *Immunity* **9**, 135–141 (1998).
14. Wiesendanger, M., Kneitz, B., Edelmann, W. & Scharff, M. D. Somatic hypermutation in MutS homologue (MSH)3⁻, MSH6⁻, and MSH3/MSH6-deficient mice reveals a role for the MSH2–MSH6 heterodimer in modulating the base substitution pattern. *J. Exp. Med.* **191**, 579–584 (2000).
15. Lindahl, T. Suppression of spontaneous mutagenesis in human cells by DNA base excision-repair. *Mutat. Res.* **462**, 129–135 (2000).
16. Sale, J. E. & Neuberger, M. S. TdT-accessible breaks are scattered over the immunoglobulin V domain in a constitutively hypermutating B cell line. *Immunity* **9**, 859–869 (1998).
17. Zeng, X. *et al.* DNA polymerase η is an A–T mutator in somatic hypermutation of immunoglobulin variable genes. *Nature Immunol.* **2**, 537–541 (2001).
18. Rogozin, I. B., Pavlov, Y. I., Bebenek, K., Matsuda, T. & Kunkel, T. A. Somatic mutation hotspots correlate with DNA polymerase ϵ error spectrum. *Nature Immunol.* **2**, 530–536 (2001).
19. Manis, J. P., Tian, M. & Alt, F. W. Mechanism and control of class-switch recombination. *Trends Immunol.* **23**, 31–39 (2002).
20. Petersen, S. *et al.* AID is required to initiate Nbs1/γ-H2AX focus formation and mutations at sites of

20. Chen, X., Kinoshita, K. & Honjo, T. Variable deletion and duplication at recombination junction ends: implication for staggered double-strand cleavage in class-switch recombination. *Proc. Natl Acad. Sci. USA* **98**, 13860–13865 (2001).
21. Miller, J. H. *Experiments in Molecular Genetics*, (Cold Spring Harbor Press, Cold Spring Harbor, 1972).
22. Mokkapatni, S. K., Fernandez de Henestrosa, A. R. & Bhagwat, A. S. *Escherichia coli* DNA glycosylase Mug: a growth-regulated enzyme required for mutation avoidance in stationary-phase cells. *Mol. Microbiol.* **41**, 1101–1111 (2001).
23. Schouten, K. A. & Weiss, B. Endonuclease V protects *Escherichia coli* against specific mutations caused by nitrous acid. *Mutat. Res.* **435**, 245–254 (1999).
24. He, B., Qing, H. & Kow, Y. W. Deoxyxanthosine in DNA is repaired by *Escherichia coli* endonuclease V. *Mutat. Res.* **459**, 109–114 (2000).
25. Betz, A. G. *et al.* Elements regulating somatic hypermutation of an immunoglobulin kappa gene: critical role for the intron enhancer/matrix attachment region. *Cell* **77**, 239–248 (1994).
26. Jarmuz, A. *et al.* An anthropoid-specific locus of orphan C to U RNA-editing enzymes on chromosome 22. *Genomics* **79**, 285–296 (2002).
27. Shen, J.-C., Rideout, W. M. & Jones, P. A. High frequency mutagenesis by a DNA methyltransferase. *Cell* **71**, 1073–1080 (1992).
28. Navaratnam, N. *et al.* *Escherichia coli* cytidine deaminase provides a molecular model for ApoB RNA editing and a mechanism for RNA substrate recognition. *J. Mol. Biol.* **275**, 695–714 (1998).
29. Selker, E. U. Premiotic instability of repeated sequences in *Neurospora crassa*. *Ann. Rev. Genet.* **24**, 579–613 (1990).
30. Jin, D. J. & Zhou, Y. N. Mutational analysis of structure-function relationship of RNA polymerase in *Escherichia coli*. *Methods Enzymol.* **273**, 300–319 (1996).

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Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast

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In eukaryotes, the DNA of the genome is packaged with histone proteins to form nucleosomal filaments, which are, in turn, folded into a series of less well understood chromatin structures¹. Post-translational modifications of histone tail domains modulate chromatin structure and gene expression^{2–4}. Of these, histone ubiquitination is poorly understood. Here we show that the ubiquitin-conjugating enzyme Rad6 (Ubc2) mediates methylation of histone H3 at lysine 4 (Lys 4) through ubiquitination of H2B at Lys 123 in yeast (*Saccharomyces cerevisiae*). Moreover, H3 (Lys 4) methylation is abolished in the H2B-K123R mutant, whereas H3-K4R retains H2B (Lys 123) ubiquitination. These data indicate a unidirectional regulatory pathway in which ubiquitination of H2B (Lys 123) is a prerequisite for H3 (Lys 4) methylation. We also show that an H2B-K123R mutation perturbs silencing at the telomere, providing functional links between Rad6-mediated H2B (Lys 123) ubiquitination, Set1-mediated H3 (Lys 4) methylation, and transcriptional silencing. Thus, these data reveal a pathway leading to gene regulation through concerted histone modifications on distinct histone tails. We refer to this as 'trans-tail' regulation of histone modification, a stated prediction of the histone code hypothesis^{5,6}.

Recent findings suggest that acetylation and methylation on the same histone tail affect each other *in vivo*^{7,8}. Given that deletion of *GCN5* results in a global decrease in H3 acetylation, and loss of *RPD3* increases the acetylation level of all four histones in regions surrounding several genomic loci in yeast⁹, we sought to determine the effect on H3 (Lys 4) methylation by deleting either of these two genes. The levels of H3 (Lys 4) methylation in *GCN5* and *RPD3* deletion strains (*gcn5Δ* and *rpd3Δ*) are similar to that of the isogenic wild-type strain (Fig. 1a, lanes 4–6). Thus, global changes in histone acetylation by deletion of *GCN5* or *RPD3* have no apparent effect on H3 (Lys 4) methylation in yeast. Our recent studies have revealed that H3 (Lys 4) methylation, and the enzyme responsible, Set1, are important for silencing at the ribosomal DNA locus^{10,11}. Because deletion of *CAC3*, *SIR2*, *SIR4* or *RAD6/UBC2* also affects rDNA and/or telomeric silencing^{12–14}, we examined whether any of these genes are required for H3 (Lys 4) methylation. Although there was no apparent effect of deleting *CAC3*, *SIR2* or *SIR4* (Fig. 1a, lanes 3, 7 and 8), deletion of *RAD6* totally abolished H3 (Lys 4) methylation (lane 2). This result indicates that Rad6 protein might have an important, but unexpected, role in regulating H3 (Lys 4) methylation in yeast.

The highly conserved *RAD6/UBC2* encodes an E2 ubiquitin-conjugating enzyme involved in many cellular processes, including DNA repair, mutagenesis induced by ultraviolet radiation, protein degradation by the amino-end rule, sporulation^{15,16} and gene silencing^{12,13}. The ubiquitin-conjugating activity of Rad6 seems to be critical for all of its biological functions, because mutation of the evolutionarily conserved ubiquitin-conjugating residue Cys 88 results in phenotypes that resemble the *rad6* null mutation¹⁷. To determine whether the enzymatic activity of Rad6 is required for H3 (Lys 4) methylation, plasmid-borne *RAD6* and its mutant alleles expressed from the endogenous promoter were introduced to the *rad6Δ* mutant YGL058W. Unlike *RAD6*, *rad6-C88A* and *rad6-Δ1–9*

(lacking the first nine amino acids) failed to restore H3 (Lys 4) methylation in *rad6Δ* (Fig. 1b, compare lanes 3, 4 and 5). In contrast, *rad6-149* and *rad6-153*, which encode for rad6 proteins lacking all, and all but four, acidic residues in the Rad6 carboxy-terminal tail domain (CTD), respectively, exhibited substantial H3 (Lys 4) methylation activity (lanes 6 and 7). It has been shown that *rad6-C88A* and *rad6-Δ1–9* mutations are defective in multi-ubiquitination and degradation of N-end rule substrates^{17,18}. Moreover, the *rad6-153* and, to a lesser degree, *rad6-149* proteins were found to retain the ability to mono-ubiquitinate histone H2A and H2B *in vitro*¹⁹. Therefore, the enzymatic activities of these *rad6* alleles towards their *in vivo* and *in vitro* substrates correlate well with the ability of each allele to restore H3 (Lys 4) methylation in *rad6Δ*. Together, these results establish that the ubiquitin-conjugating activity of Rad6 is required for H3 (Lys 4) methylation.

Genetic complementation analyses revealed that the human *RAD6* homologues *HHR6A* and *HHR6B* were able to carry out the Rad6-mediated DNA repair and ultraviolet-induced mutagenesis functions in yeast²⁰. To determine whether the mouse *RAD6* homologues *HR6A* and *HR6B* (identical to human) can also carry out the Rad6-mediated H3 (Lys 4) methylation in yeast, *HR6A*-Flag and *HR6B*-Flag were expressed from the yeast *ADHI* promoter in the *rad6Δ* strain YGL058W. The levels of Lys 4-methylated H3 in *rad6Δ* containing *RAD6*-Flag or its mouse homologues were essentially the same (Fig. 1c, lanes 1, 3 and 4). Thus, the function of Rad6 in mediating H3 (Lys 4) methylation is highly conserved.

Rad6 mediates its DNA repair function through interaction with Rad18, a single-stranded DNA-binding protein that targets Rad6–Rad18 complexes to damaged DNA²¹. For its role in N-end rule-dependent protein degradation, Rad6 physically associates with the Ubr1 ubiquitin protein ligase (E3)^{18,22}. To determine whether H3 (Lys 4) methylation is modulated through the pathway of Rad6–Rad18-mediated DNA repair or Rad6–Ubr1-dependent protein degradation, we examined the requirement for *RAD18* and *UBR1* in regulating H3 (Lys 4) methylation. Unlike *rad6Δ*, both *rad18Δ* and *ubr1Δ* mutants displayed wild-type levels of H3 (Lys 4) methylation (Fig. 1d, compare lanes 2, 3 and 4). Thus, these results indicate that both Rad6–Rad18- and Rad6–Ubr1-mediated pathways are not involved in regulating H3 (Lys 4) methylation and suggest a role for Rad6 in this histone modification process.

As well as the Rad6-mediated cellular processes described above, Rad6 ubiquitinates histones H2A and H2B *in vitro*^{19,23}. Moreover, Rad6 is the major cellular activity that ubiquitinates H2B at Lys 123 in yeast²⁴. We therefore asked whether the conserved ubiquitination sites of H2B and/or H2A are important for H3 (Lys 4) methylation. The level of Lys 4-methylated H3 in the H2A-4K/R mutant, which contains lysine-to-arginine substitutions at the conserved (Lys 119) and other potential ubiquitination sites (Lys 120, 123 and 126) in the C terminus of H2A, is indistinguishable from that in an isogenic wild-type strain (Fig. 2a, compare lanes 1 and 2). In striking contrast, H3 (Lys 4) methylation is totally abolished in the H2B-K123R single (lane 4) and H2B-K123R/H2A-4K/R double mutants (lane 3). Thus, only Lys 123 of H2B is critical for H3 (Lys 4) methylation. (An identical result was also obtained with the H2B-K123A mutant (data not shown).) Because expression levels of *SET1* and its associated genes²⁵ are essentially the same in the isogenic wild-type, *rad6Δ* and H2B-K123R strains (Fig. 2b and Supplementary Information), the diminished H3 (Lys 4) methylation in *rad6Δ* and H2B-K123R mutants cannot be attributed to loss of the Set1 protein complex required for this modification. Together, these results strongly argue that Rad6 exerts its effect on H3 (Lys 4) methylation through the ubiquitination of H2B.

It is curious that the *rad6-149* mutant retains substantial H3 (Lys 4) methylation activity (Fig. 1b, lane 6) because it was previously reported that the Lys 123-ubiquitinated form of H2B (uH2B) was not detected in this mutant²⁴. However, it has been suggested that the *rad6-149* protein might maintain low, but sufficient, levels of

uH2B to support normal cell growth²⁴. If this were the case, we reasoned that overexpression of *rad6-149* might lead to enhanced detection of uH2B. To test this possibility, levels of uH2B and Lys 4-methylated H3 were examined in the *rad6Δ* mutant YZS279 carrying low- (*CEN*) or high-copy (*2μ*) *RAD6* alleles. For detection of uH2B, the endogenous H2B in YZS279 was replaced by the plasmid-borne Flag-tagged H2B (Flag-H2B). Two bands were specifically detected by the Flag antibody in the *RAD6* cellular lysates that correspond to the expected positions of Flag-H2B and Flag-uH2B (Fig. 2c, lane 2). Only the faster-migrating band, not the slower-migrating band, was detected in the Flag-H2B-K123R mutant (lane 1). We therefore conclude that the upper and lower bands represent the ubiquitinated (Flag-uH2B) and unmodified Flag-H2B, respectively. In keeping with the previous study²⁴, Flag-uH2B was not detected in strains containing low-copy *rad6-C88A* or *rad6-149* alleles (lanes 3 and 4), and, like our earlier data (Fig. 1b), only *rad6-149* exhibits, albeit to a lesser degree, H3 (Lys 4) methylation in

these two strains. In contrast, Flag-uH2B was elevated to a detectable level in the same *rad6Δ* mutant carrying high-copy *rad6-149* plasmid (compare lanes 4 and 9), and H3 (Lys 4) methylation was correspondingly enhanced. This result establishes that the *rad6-149* protein retains a low level of H2B (Lys 123) ubiquitination activity, which, when overexpressed, enhances both H2B (Lys 123) ubiquitination and H3 (Lys 4) methylation *in vivo*.

The first nine amino acids of Rad6 are important for the association of Rad6 with Ubr1 (ref. 18), and our earlier data demonstrate that the Rad6-Ubr1-mediated pathway is not involved in H3 (Lys 4) methylation (Fig. 1d). Thus, the complete absence of

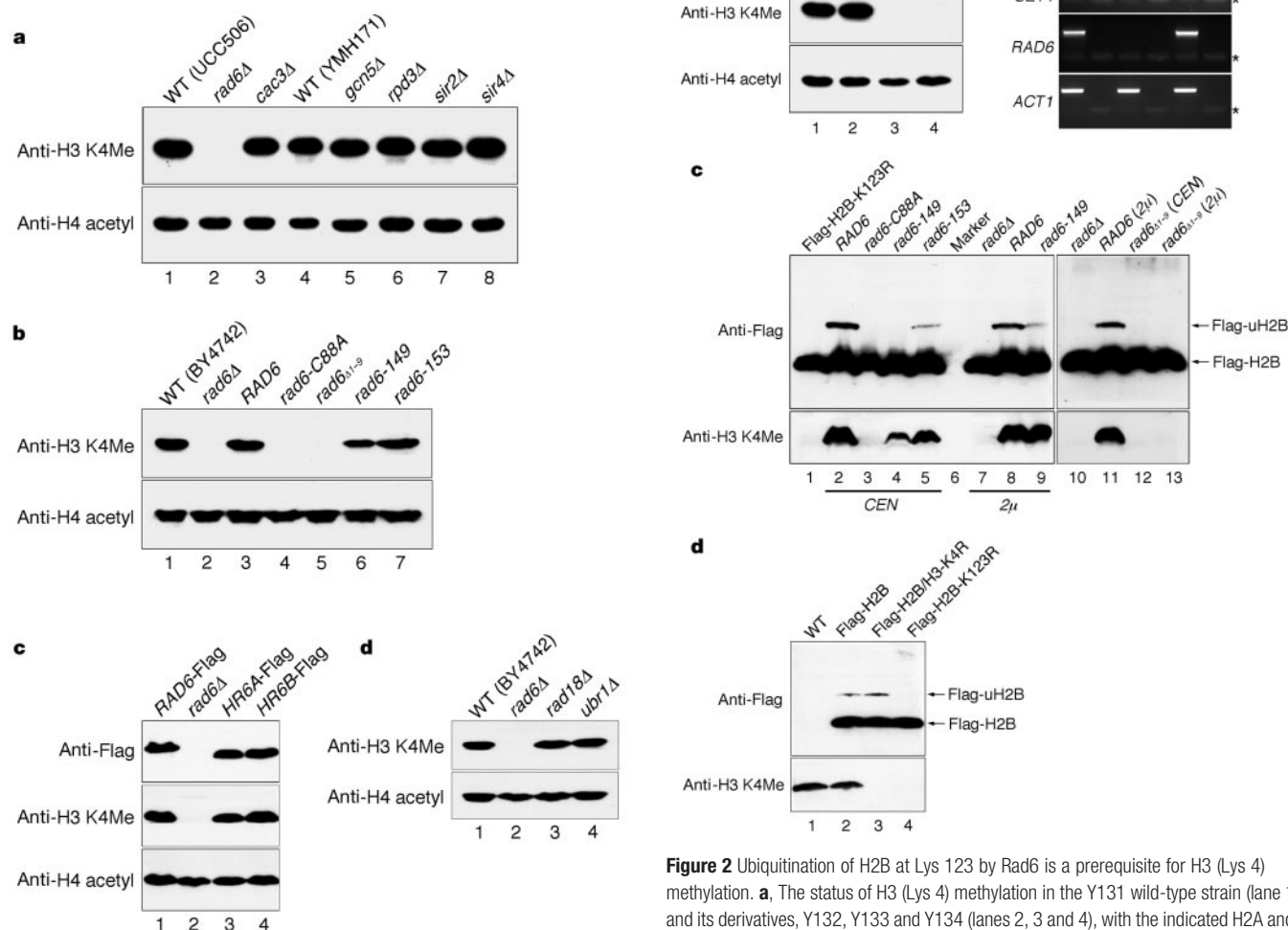


Figure 1 The ubiquitin-conjugating enzyme Rad6 is important for H3 (Lys 4) methylation. **a**, Yeast whole-cell extracts (WCEs) prepared from the indicated wild-type (WT) strains and their derivatives were analysed for H3 (Lys 4) methylation by western blot using H3 K4Me antibody. The H4 acetyl antibody was used to monitor the histone loading. **b**, WCEs isolated from BY4742 (wild type) and its isogenic *rad6Δ* strain (YGL058W) containing the YCp50 vector (lane 2), *RAD6* or its mutant alleles (lanes 3–7) were analysed for H3 (Lys 4) methylation as in **a**. **c**, WCEs of the *rad6Δ* mutant (YGL058W) containing the p416 ADH vector (lane 2), the Flag-tagged *RAD6* or its mouse homologues *HR6A* and *HR6B* (lanes 1, 3 and 4) driven by the *ADH1* promoter, were assessed for H3 (Lys 4) methylation as in **a**. The expression of the indicated Flag-tagged proteins was monitored using Flag antibody. **d**, The levels of H3 (Lys 4) methylation in the wild-type BY4742, *rad6Δ* (YGL058W), *rad18Δ* (YCR066W) and *ubr1Δ* (YGR184C) strains were analysed as in **a**.

Figure 2 Ubiquitination of H2B at Lys 123 by Rad6 is a prerequisite for H3 (Lys 4) methylation. **a**, The status of H3 (Lys 4) methylation in the Y131 wild-type strain (lane 1) and its derivatives, Y132, Y133 and Y134 (lanes 2, 3 and 4), with the indicated H2A and/or H2B mutant alleles were assessed as in Fig. 1. **b**, Expression levels of the indicated genes in the isogenic wild-type, *rad6Δ* and H2B-K123R strains were analysed by PCR with or without reverse transcriptase (see Supplementary Information for expression data of other *SET1*-associated genes). The *ACT1* PCR products were used as loading controls. RTase, reverse transcriptase; asterisk, unincorporated primer. **c**, The presence of Flag-H2B and Flag-uH2B in lysates (see Methods) prepared from YZS279 (Flag-H2B, *rad6Δ*) and its derivatives with the indicated low- (*CEN*) or high-copy (*2μ*) *RAD6* alleles or a *2μ* vector (lanes 7 and 10) was analysed by western blotting using Flag antibody. The levels of H3 (Lys 4) methylation in the same set of lysates were also assessed. **d**, Flag-uH2B and Lys 4-methylated H3 in the YZS266 wild-type strain containing vector (lane 1) or pZS139 (Flag-H2B) (lane 2), and in the isogenic H3-K4R mutant YZS267 with pZS139 (lane 3), were analysed as in **c**. Lysates from the Flag-H2B-K123R mutants, YZS277 and YZS246, were used as negative controls for **c** and **d**, respectively.

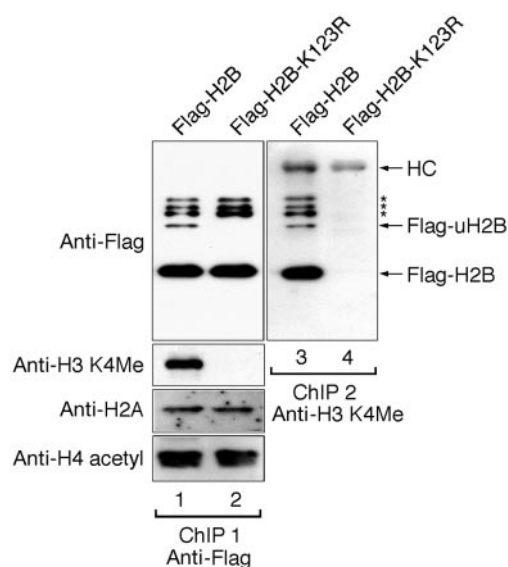


Figure 3 Ubiquitinated Flag-H2B is associated with chromatin containing methylated (Lys 4) H3. Histone proteins in the chromatin immunoprecipitate obtained from YZS245 (Flag-H2B) and YZS246 (Flag-H2B-K123R) strains were analysed by western blot using Flag and the indicated histone antibodies. A second round of ChIP was performed using H3 K4Me antibody and the precipitate recovered from the first round of ChIP. The presence of Flag-H2B and Flag-uH2B in the resulting second-round ChIP were also assessed. HC, immunoglobulin heavy chain; asterisk, histone proteins remain cross-linked to Flag-H2B after SDS sample buffer treatment (see Methods); ChIP 1 and ChIP 2 are the first and second round of ChIP assays, respectively.

H3 (Lys 4) methylation in the *rad6 Δ 1-9* mutant was unexpected (Fig. 1b, lane 5). This effect might be due to insufficient Rad6 protein when *rad6 Δ 1-9* is present on a low-copy plasmid. However, unlike *rad6-149*, neither uH2B nor Lys 4-methylated H3 was detected when *rad6 Δ 1-9* was overexpressed from a high-copy plasmid (Fig. 2c, lane 13). This result indicates that the N terminus of Rad6 regulates H2B (Lys 123) ubiquitination and H3 (Lys 4) methylation in a Ubr1-independent manner. Together, these data reinforce the argument that Rad6 exerts its effect on H3 (Lys 4) methylation through ubiquitination of H2B.

To determine whether methylation of H3 Lys 4 and ubiquitination of H2B Lys 123 are essential to each other, we investigated whether H2B Lys 123 is ubiquitinated in the H3-K4R mutant. Flag-uH2B was detected in both wild-type and H3-K4R strains (Fig. 2d, lanes 2 and 3). Thus, H3 (Lys 4) methylation is not required for H2B (Lys 123) ubiquitination.

Although we established that H2B ubiquitination was essential for H3 (Lys 4) methylation, it was unclear whether uH2B mediated this process in a chromatin-bound context in yeast. To address this, we examined uH2B and H3 (Lys 4) methylation in a chromatin context using chromatin immunoprecipitation (ChIP) assays.

Initially, chromatin prepared from Flag-H2B and Flag-H2B-K123R strains was bound to anti-Flag M2 affinity resin and eluted with the Flag peptide. The presence of histone proteins in the released chromatin was examined. Whereas equivalent amounts of unmodified Flag-H2B, H2A and acetylated H4 were detected in the chromatin immunoprecipitate from both strains, neither Flag-uH2B nor Lys 4-methylated H3 was detected in the precipitate from the Flag-H2B-K123R mutant (Fig. 3, compare lanes 1 and 2). Thus, again, these results confirm that H2B Lys 123 is critical for H3 (Lys 4) methylation, and suggest that Flag-uH2B is assembled into chromatin. However, it remains a formal but remote possibility that Flag-uH2B exists in a 'free', non-chromatin-bound form, instead of being associated with the chromatin containing methylated (Lys 4) H3. Currently, there are no antibodies that are specific for uH2B, so conventional (histone:DNA) ChIP assays cannot be used to assess the chromatin-bound nature of uH2B. Instead, we employed the H3 K4Me antibody to perform a second round of ChIP using the precipitate recovered from the first round ChIP, and analysed the resulting second-round precipitate for the presence of Flag-uH2B. Flag-uH2B was easily detected in the precipitate recovered from the Flag-H2B strain (Fig. 3, lane 3), demonstrating that Flag-uH2B is associated with chromatin containing methylated (Lys 4) H3.

Rad6 has been shown to regulate transcriptional silencing at all known silencing loci (telomere, *HM* and *rDNA*) in yeast, and, importantly, its ubiquitin-conjugating activity is essential for this process^{12,13}. As summarized in Table 1, the ability of each of the *RAD6* alleles to ubiquitinate H2B (Lys 123) correlates well with its ability to modulate transcriptional silencing and Set1-mediated H3 (Lys 4) methylation functions^{10,11}. This correlation is further supported by the findings that both Rad6-Rad18- and Rad6-Ubr1-mediated pathways, which are not involved in H3 (Lys 4) methylation (Fig. 1d), are not required for Rad6-mediated transcriptional silencing^{12,13}.

Our data document a regulatory pathway wherein Rad6-mediated H2B (Lys 123) ubiquitination regulates H3 (Lys 4) methylation that, in turn, regulates silencing. If correct, we predicted that an H2B-K123R mutation would be sufficient to confer a silencing defect. To test this, expression of the *URA3* gene, located at the left-end telomere of chromosome VII (*URA3-TEL* (VII-L)), was assessed in the H2B-K123R mutant. In contrast to the isogenic wild-type strain (*URA3-TEL*), both *sir2 Δ* (a gene required for telomeric silencing) and H2B-K123R mutants carrying *URA3-TEL* failed to grow on FOA medium (Fig. 4, compare rows 2, 3 and 4). Thus, this result establishes that H2B Lys 123 is essential for telomeric silencing in yeast.

We wondered about the molecular basis for this concerted series of enzyme reactions. Recent analyses of the crystal structure of yeast nucleosome core particles revealed that the C-terminal helix of H2B is involved in internucleosomal interactions²⁶. Thus, ubiquitination of Lys 123 at the H2B C terminus might disrupt these internucleosomal interactions, and thereby alter local or global chromatin folding²⁶. We note that the amount of uH2B in bulk preparations of

Table 1 Phenotypes associated with alleles

Allele	H2B (Lys 123) ubiquitination*	H3 (Lys 4) methylation†	Transcriptional silencing‡
<i>RAD6</i>	+	+	+
<i>rad6Δ</i>	-	-	-
<i>rad6-C88A</i>	-	-	-
<i>rad6Δ1-9</i>	-	-	-
<i>rad6-149</i>	+/-	+/-	+\$
<i>rad6-153</i>	+/-	+/-	+
H2B-K123R mutant	-	-	-

* This study and ref. 24.

† This study.

‡ At telomere, *HM* and *rDNA* loci^{12,13}.

\$ Tested at *rDNA* only¹³.

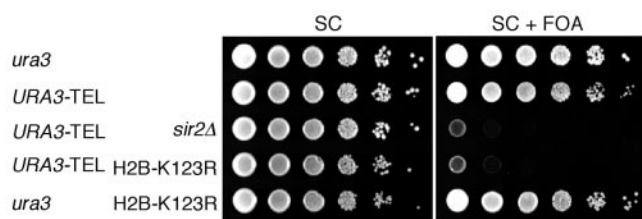


Figure 4 H2B Lys 123 is important for telomeric silencing. Expression of the telomere-linked *URA3* gene (*URA3-TEL*) in the isogenic YZS273 (*URA3-TEL*), YZS274 (*URA3-TEL* H2B-K123R) and YZS275 (*URA3-TEL sir2Δ*) strains was assayed on the synthetic complete medium containing FOA (SC + FOA). YZS276 (*ura3*) and YZS277 (*ura3* H2B-K123R) were used as controls for growth on the same medium. Impaired growth on FOA medium indicates diminished silencing of *URA3*.

yeast H2B is low (<5%) (this study, and M. A. Osley, personal communication), whereas the steady-state amount of Lys 4-methylated H3 is considerably higher (~35%)²⁷. Thus, it is unlikely that H3 (Lys 4) methylation is strictly dependent on ubiquitination of H2B Lys 123 on the same nucleosome. Instead, we favour the view that a single, bulky ubiquitin adduct may act more as a 'wedge' to open stretches of chromatin and, in turn, allow access for the Set1 histone methyltransferase complex to the relevant chromatin domains. Although Lys 4-methylated H3 is generally regarded as an 'activating mark' for chromatin remodelling^{7,8}, it remains unclear how the pathway described here modulates transcriptional silencing. For example, we cannot rule out the possibility that the observed effect on silencing results from an indirect mechanism. In keeping with our results, however, a recent study indicates that both Rad6 and H3B K123 are required for the repression of *ARG1* transcription in yeast³⁰.

In summary, our data suggest that Rad6 regulates transcriptional silencing through a concerted series of histone modification events. To our knowledge, this is the first demonstration of a cellular process that is regulated by a unidirectional 'trans-tail' histone modification where a covalent modification on one histone tail is dependent on another modification on a different histone tail. □

Methods

Yeast strains

Yeast strains YMH270 (*rad6Δ::LEU2*), YMH276 (*cac3Δ::LEU2*) and YMH405 (*rad6Δ::LEU2*), and their corresponding parent strains, YMH171 and UCC506, were described previously^{28,29}. BY4742 and its derivatives, YGL058W (*rad6Δ*), YCR066W (*rad18Δ*) and YGR184C (*ubr1Δ*), were obtained from Research Genetics. Strains Y132 (*hta1-4K/R-HTB1* (2 μ *URA3*)), Y133 (*HTA1-htb1-K123R* (2 μ *URA3*)) and Y134 (*hta1-4K/R-htb1-K123R* (2 μ *URA3*)), which are isogenic to Y131 (*hta1-htb1Δ::LEU2* *htb2Δ* *HTA1-HTB1* (2 μ *URA3*)), were described previously²⁴ (obtained from M. A. Osley). Strains YZS245 (pZS139 *HTA1-Flag-HTB1* 2 μ *HIS3*), YZS246 (pZS140 *HTA1-Flag-htb1-K123R* 2 μ *HIS3*), YZS276 (pZS145 *HTA1-Flag-HTB1* *CEN HIS3*), YZS277 (pZS146 *HTA1-Flag-htb1-K123R* *CEN HIS3*) and YZS280 (pZS147 *HTA1-Flag-htb1-K123A* *CEN HIS3*) are derived from Y131. YZS266 (pZS136 *HHT2-HHF2* *CEN TRP1*) and YZS267 (pZS138 *hht2-K4R-HHF2* *CEN TRP1*) were derived from MSY421 (ref. 11) (*hht1-hhf1Δ hht2-hhf2Δ* pMS329 (*HHT1-HHF1* *CEN URA3*)) (obtained from M. Smith). YZS272 (pZS144 *HTA1-Flag-HTB1* *CEN TRP1*) *URA3-TEL*, a derivative of Y131, was generated by integrating the *URA3* gene at the left-end telomere of chromosome VII as described²⁹. Strains YZS273 (pZS145 *HTA1-Flag-HTB1* *CEN HIS3*) *URA3-TEL*, YZS274 (pZS146 *HTA1-Flag-htb1-K123R* *CEN HIS3*) *URA3-TEL* and YZS275 (pZS145 *HTA1-Flag-HTB1* *CEN HIS3*) *URA3-TEL sir2Δ::TRP1*) are derived from YZS272. YZS279 (pZS145 *HTA1-Flag-HTB1* *CEN HIS3*) *rad6Δ::ura3*) is a derivative of YZS276.

DNA constructs

All of the YCp50 (*CEN*, *URA3*)-based *RAD6* and its mutant alleles shown in Fig. 1b were described previously^{17,18} (obtained from M. Bryk and F. Winston). The N-terminal Flag-tagged H2B construct, pZS139 (*HTA1-Flag-HTB1*), was generated by site-directed polymerase chain reaction (PCR) mutagenesis using pZS137 (*HTA1-HTB1*, 2 μ , *HIS3*) as template. The pZS140 (*HTA1-Flag-htb1-K123R*) and pZS138 (*hht2-K4R-HHF2*) constructs were also generated in the same way using pZS139 and pZS136 (*HHT2-HHF2*, *CEN*, *TRP1*) as templates, respectively. pZS144 (*HTA1-Flag-HTB1*, *CEN*, *TRP1*) was generated by transferring *SpeI*–*SacII* fragment encompassing *HTA1-Flag-HTB1* from

pZS139 to the same sites of pZS314 (*CEN*, *TRP1*). pZS145 (*HTA1-Flag-HTB1*, *CEN*, *HIS3*) and pZS146 (*HTA1-Flag-htb1-K123R*, *CEN*, *HIS3*) were generated by transferring *SpeI*–*SacII* fragments of pZS139 and pZS140 to pZS313 (*CEN*, *HIS3*), respectively.

Western blot analysis

Whole-cell extracts were prepared and analysed on 15% SDS–polyacrylamide gels as described¹¹. For the preservation and detection of ubiquitinated Flag-H2B, cells (~1 $\times 10^7$ cells per ml) from overnight 5-ml cultures were collected, washed and immediately boiled in 70 μ l of 1 $\times$ SDS sample buffer. After centrifugation (14000g for 5 min), ~10 μ l of the resulting lysates was subjected to western analysis using monoclonal Flag antibody. In this study, the H4 acetyl antibody was used for a loading control because *rad6Δ* and H2B-K123R mutations do not affect bulk levels of H4 acetylation (data not shown). Both the H3 K4Me and H4 acetyl antibodies (rabbit) were used as described¹¹. Mouse monoclonal Flag antibody (M2, Sigma) was used at 1 μ g ml⁻¹. H2A antibody (Upstate Biotechnology), which recognizes residues 88–97 in the histone fold domain of human H2A, was used at a 1:250 dilution.

PCR with reverse transcription (RT–PCR)

We subjected 100 μ g of total RNA isolated from each of the isogenic strains, YZS276 (wild type), YZS277 (H2B-K123R) and YZS279 (*rad6Δ*), to poly A⁺ messenger RNA isolation using Oligotex mRNA Kit (Qiagen). One-tenth of the resulting mRNA from each strain was applied to synthesize complementary DNA using the ThermoScript RT–PCR System (Invitrogen). We subjected 1 μ l of cDNA from each reaction (20 μ l total) to PCR amplification (30 cycles) using primers specific to the indicated genes. One-fifth of the PCR product was analysed on 1.5% agarose gel.

ChIP assay

Preparation and immunoprecipitation of formaldehyde cross-linked chromatin were performed as described¹¹. The formaldehyde cross-linked chromatin isolated from YZS245 (Flag-H2B) and YZS246 (Flag-H2B-K123R) strains were immunoprecipitated using Flag M2 affinity resin (Sigma) and eluted with the Flag peptide (4 mg ml⁻¹) (Sigma). The presence of histone proteins in the chromatin immunoprecipitate was assessed by boiling the eluted chromatin in 1 $\times$ SDS sample buffer for 5 min and analysed by western blot using Flag and the indicated histone antibodies. H3 K4Me antibody and the precipitate recovered from the first round ChIP were used to perform a second round of ChIP. To detect Flag-H2B and Flag-uH2B, the chromatin-bound protein A Sepharose was directly boiled in 1 $\times$ SDS sample buffer and resolved by 15% SDS–polyacrylamide gel electrophoresis for western analysis using Flag antibody.

Telomeric silencing assay

Silencing of the *URA3* gene integrated at the left-end telomere of chromosome VII (*URA3-TEL* (VII-L)) was assayed as described previously²⁸.

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- Kornberg, R. D. & Lorch, Y. Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome. *Cell* **98**, 285–294 (1999).
- van Holde, K. E. in *Chromatin* (ed. Rich, A.) 111–148 (Springer, New York, 1989).
- Urnov, F. D. & Wolffe, A. P. Above and within the genome: epigenetics past and present. *J. Mammary Gland Biol. Neoplasia* **6**, 153–167 (2001).
- Workman, J. L. & Kingston, R. E. Alteration of nucleosome structure as a mechanism of transcriptional regulation. *Annu. Rev. Biochem.* **4**, 545–579 (1998).
- Strahl, B. D. & Allis, C. D. The language of covalent histone modifications. *Nature* **403**, 41–45 (2000).
- Turner, B. M. Histone acetylation and an epigenetic code. *Bioessays* **22**, 836–845 (2000).
- Jenuwein, T. & Allis, C. D. Translating the histone code. *Science* **293**, 1074–1080 (2001).
- Zhang, Y. & Reinberg, D. Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. *Genes Dev.* **15**, 2343–2360 (2001).
- Vogelauer, M., Wu, J., Suka, N. & Grunstein, M. Global histone acetylation and deacetylation in yeast. *Nature* **408**, 495–498 (2000).
- Bryk, M. *et al.* Evidence that Set1, a factor required for methylation of histone H3, regulates rDNA silencing in *S. cerevisiae* by a Sir2-independent mechanism. *Curr. Biol.* **12**, 165–170 (2002).
- Briggs, S. D. *et al.* Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in *Saccharomyces cerevisiae*. *Genes Dev.* **15**, 3286–3295 (2001).
- Huang, H., Kahana, A., Gottschling, D. E., Prakash, L. & Liebman, S. W. The ubiquitin-conjugating enzyme Rad6 (Ubc2) is required for silencing in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **17**, 6693–6699 (1997).
- Bryk, M. *et al.* Transcriptional silencing of Ty1 elements in the RDN1 locus of yeast. *Genes Dev.* **11**, 255–269 (1997).
- Smith, J. S., Caputo, E. & Boeke, J. D. A genetic screen for ribosomal DNA silencing defects identifies multiple DNA replication and chromatin-modulating factors. *Mol. Cell. Biol.* **19**, 3184–3197 (1999).
- Prakash, S., Sung, P. & Prakash, L. DNA repair genes and proteins of *Saccharomyces cerevisiae*. *Annu. Rev. Genet.* **27**, 33–70 (1993).
- Varshavsky, A. The N-end rule. *Cold Spring Harb. Symp. Quant. Biol.* **60**, 461–478 (1995).
- Sung, P., Prakash, S. & Prakash, L. Mutation of cysteine-88 in the *Saccharomyces cerevisiae* RAD6 protein abolishes its ubiquitin-conjugating activity and its various biological functions. *Proc. Natl Acad. Sci. USA* **87**, 2695–2699 (1990).
- Watkins, J. E., Sung, P., Prakash, S. & Prakash, L. The extremely conserved amino terminus of RAD6 ubiquitin-conjugating enzyme is essential for amino-end rule-dependent protein degradation. *Genes Dev.* **7**, 250–261 (1993).

19. Sung, P., Prakash, S. & Prakash, L. The RAD6 protein of *Saccharomyces cerevisiae* polyubiquitinates histones, and its acidic domain mediates this activity. *Genes Dev.* **2**, 1476–1485 (1988).
20. Koken, M. H. *et al.* Structural and functional conservation of two human homologs of the yeast DNA repair gene RAD6. *Proc. Natl Acad. Sci. USA* **88**, 8865–8869 (1991).
21. Bailly, V., Prakash, S. & Prakash, L. Domains required for dimerization of yeast Rad6 ubiquitin-conjugating enzyme and Rad18 DNA binding protein. *Mol. Cell. Biol.* **17**, 4536–4543 (1997).
22. Madura, K., Dohmen, R. J. & Varshavsky, A. N-recognin/Ubc2 interactions in the N-end rule pathway. *J. Biol. Chem.* **268**, 12046–12054 (1993).
23. Jentsch, S., McGrath, J. P. & Varshavsky, A. The yeast DNA repair gene RAD6 encodes a ubiquitin-conjugating enzyme. *Nature* **329**, 131–134 (1987).
24. Robzyk, K., Recht, J. & Osley, M. A. Rad6-dependent ubiquitination of histone H2B in yeast. *Science* **287**, 501–504 (2000).
25. Roguiev, A. *et al.* The *Saccharomyces cerevisiae* Set1 complex includes an Ash2 homologue and methylates histone 3 lysine 4. *EMBO J.* **20**, 7137–7148 (2001).
26. White, C. L., Suto, R. K. & Luger, K. Structure of the yeast nucleosome core particle reveals fundamental changes in internucleosome interactions. *EMBO J.* **20**, 5207–5218 (2001).
27. Strahl, B. D., Ohba, R., Cook, R. G. & Allis, C. D. Methylation of histone H3 at lysine 4 is highly conserved and correlates with transcriptionally active nuclei in *Tetrahymena*. *Proc. Natl Acad. Sci. USA* **96**, 14967–14972 (1999).
28. Sun, Z. W. & Hampsey, M. A general requirement for the Sin3–Rpd3 histone deacetylase complex in regulating silencing in *Saccharomyces cerevisiae*. *Genetics* **152**, 921–932 (1999).
29. Renaud, H. *et al.* Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by SIR3 dosage. *Genes Dev.* **7**, 1133–1145 (1993).
30. Turner, S. D. *et al.* The E2 ubiquitin conjugase Rad6 is required for the ArgR/Mcm1 repression of ARG1 transcription. *Mol. Cell. Biol.* **22**, 4011–4019 (2002).

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The authors declare that they have no competing financial interests.

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corrigenda

BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes

Philippe Bouillet, Jared F. Purton, Dale I. Godfrey, Li-Chen Zhang, Leigh Coultas, Hamsa Puthalakath, Marc Pellegrini, Suzanne Cory, Jerry M. Adams & Andreas Strasser

Nature **415**, 922–926 (2002).

The number of CD4⁺ CD8⁺ thymocytes shown in Fig. 1a of this Letter should be $\times 10^7$, and not $\times 10^6$ as published. This error does not affect our conclusions. □

Sudden aseismic fault slip on the south flank of Kilauea volcano

Peter Cervelli, Paul Segall, Kaj Johnson, Michael Lisowski & Asta Miklius

Nature **415**, 1014–1018 (2002).

This Letter contains two errors. First, the units of pressure for the total surface load are incorrectly given as MPa instead of bars (1 bar = 0.1 MPa). Second, in the second paragraph on page 1016, ΔH was incorrectly defined as “the change in water table height” instead of “the amount of rainfall as measured by a rain gauge”. □

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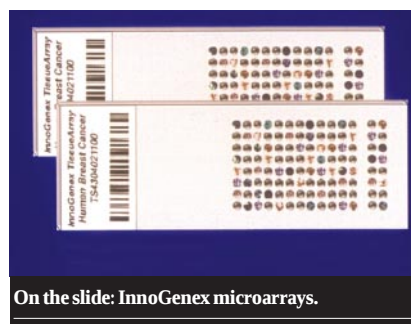
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On the slide: InnoGenex microarrays.



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The chips are down

Investing in science is a bit like playing roulette — you can spread your chips on several numbers, anticipating modest returns, or you can stack them high on a few, in hopes of a big payoff. Several Norwegian biologists had hoped that their government, when deciding how to award 140 million kroner (US\$18.7 million) a year over 10 years for new scientific initiatives, would wager in favour of biotech (see *Naturejobs* 2 May, 2002). However, with the announcement last month of 13 diverse 'Centres of Excellence' — only one of which is explicitly linked to biomedicine — the country seems instead to be hedging its bets.

The sole biomedical winner has mixed feelings about his largesse. Ole Petter Ottersen, who received the nod to establish the Centre for Molecular Biology and Neuroscience at the University of Oslo, says he is happy to receive the funding, but adds that his joy is tempered by the lack of investment elsewhere in his field. "Biomedicine ought to have several centres," he says. Two more of the 13 centres have biological components — the Agricultural University of Norway (NLH) has been awarded an aquaculture centre, and the International Centre for the Biology of Memory will be hosted at the Norwegian University of Science and Technology at Trondheim.

What does this distribution of limited resources mean in terms of recruitment? Norway — which has a goal of attracting more scientists from abroad — will need to draw from more diverse backgrounds than if it had invested only in biology. This probably comes as positive news for scientists looking for work in fields in which Norway already has a history of research, such as climate change and geosciences. Bergen, for example, will host new centres in both of these areas. But for Norwegian biologists, the news means that the country will have a harder time reaching the 'critical mass' that is necessary to compete for talent with the more established biotech hubs. Indeed, it seems that this is a game the government is reluctant to play.

Paul Smaglik

Naturejobs editor



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The biotech scene, although volatile, will continue to provide jobs. But potential employees should prepare for a bumpy ride, say Paul Smaglik and Adam Smith.

B iotechnology and job security look as though they're mutually exclusive, judging by the business news of the past few months. In Seattle, for example, Immunex employees are concerned about their future as the company is taken over by Amgen of Thousand Oaks, California. In Cambridge, Massachusetts, Variagenics recently reduced its workforce by about a third.

But despite turmoil in individual companies, there is inherent stability, even growth, built into biotech, say recruiters and analysts. According to the Biotechnology Industry Organization (BIO), the industry has more than tripled in size since 1992, with 174,000 people employed in the United States alone, and BIO predicts steady growth over the next 10 years.

To Steve Burrill, head of San Francisco-based life-sciences venture capitalists Burrill & Co., the current downturn in the biotech stock market, slowdown in venture-capital investment and consolidation of companies is nothing new — just another bump in the ride. "In the aggregate, I think growth is very robust," says Burrill. Scientists laid off from biotech companies "seem to be absorbed relatively quickly" into others.

The trick is learning to ride the roller-coaster. Survival depends on resigning oneself to a degree of mobility. Updating skills, tapping into formal and informal networks and relocating to a 'hub' area with a high concentration of biotech businesses of varying sizes and stages of development are all key ways to become mobile and stay employed, say recruiters.

SETTING SKILLS

Peter Brixius, vice-president of Kelly Scientific Resources, an international recruitment agency, says that the people most in demand tend to have multiple skills. Training in shortage areas such as clinical-trials management and regulatory affairs help scientists to break into the biotech sector. And, should the first company they sign on with falter, these skills all but guarantee a new position somewhere else.

Smaller biotech companies are famous for demanding that employees wear several hats — scientific and administrative. Embracing a variety of experiences with a small company can help a scientist to find a more distinct role in a larger company once the company's circumstances or their own ambition determines that it is time to move on, says Brixius.

James Young, head of the scientific recruitment agency Young International Group, based in Philadelphia, is now seeing more scientists attuned to developing their skills. "Career progression is the primary focus," he says. "Academics are more likely to go to biotechs because it is an easier transition. In many cases they find the technology and research intriguing, and perhaps more freedom devoid of bureaucracy than they might have in a large pharma organization."

Young agrees with Brixius that scientists who already have experience in getting a drug through the development pipeline and into the market — or at least into clinical trials — are in demand. Skill here can be essential for a company's success or failure.

Academia to industry and back

When the number of university positions on offer declined in the 1980s, Ole Bjerrum decided that, rather than possibly wait years for a tenure-track position, he would go into industry.

He left the University of Copenhagen and landed a job at Novo Nordisk, a local pharmaceutical company, and was soon caught up in the drug-discovery buzz. "At first it was very exciting," he says, "because there were so many opportunities, so much money, so many projects, assessment of success and so on." But eventually reality set in and some projects were closed down. "After the third or fourth time, it's more difficult to find the

necessary enthusiasm."

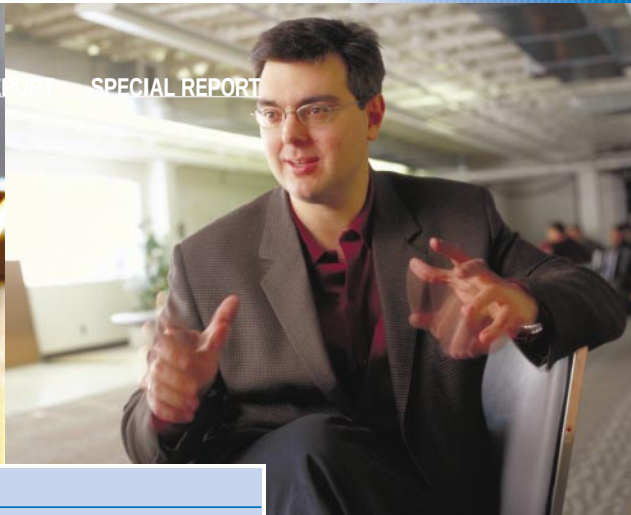
So he switched to administering academic-industrial liaisons in Novo. But when he turned 57 he realized that he had to make another decision, as he had only seven years left before retiring. "In academia, I thought, I'd have 10–12 more years to go and could give all this understanding I have picked up in industry back to the students," he says.

So he took up a professorship at the Royal Danish School of Pharmacy, which he's found satisfying, apart from the teaching load. Most of his students are thinking of going into industry, often for the same reasons that he left academia.

A.S.

Ole Bjerrum returned to academia after a spell in industry.





Quick change

He points to ImClone, a New York-based company whose fortune is uncertain because its clinical trials were misinterpreted (see *Nature* 417, 584–586; 2002). “The ImClone débâcle shows what can result from a flawed development plan,” Young says.

NETWORK

Janice Bourque, president and chief executive of the Massachusetts Biotechnology Council, says that formal networking can provide a cushion. The council helps companies who are cutting jobs put together a binder of the laid-off employees’ resumé and share them with other companies, sometimes creating informal job fairs on site.

When Repligen, with 300 employees, had to reorganize about 10 years ago, cutting all but eight or so positions, the binder approach helped most workers to find jobs with other companies in the area such as Genzyme and Biogen.

Informal networks are active in the Boston/Cambridge area, with recruiters having a good sense of the skills that different members of the biotech community possess. “Companies are very aggressive,” Bourque says. “If they need another employee, they’ll go out and get one. If it comes from another company, it comes from another company.”

LOCATION

Of course, networking is easier when you’re in an area with lots of biotech firms — preferably of different sorts and focusing on diverse technologies and targets. That sort of environment has allowed areas such as San Francisco and both Cambridges to grow.

But success can also work against a region, says Bill Domann, founder of the California-based Domann Organization, an executive-level scientific recruitment firm. “It’s difficult to recruit people into the Bay Area because of the cost of living.” Boston and Cambridge, UK are beginning to have the same problem.

Domann says that junior scientists tend to be mobile within a region, but that more senior scientists and management are more willing to move from region to region, given the right opportunity. But if they do move, they favour regions with lots of activity. Otherwise, if the company isn’t around in two or three years, “there aren’t many other alternatives,” he says.

After the Oakland, California-based bioinformatics company DoubleTwist folded in March, its chief executive, Rob Williamson, thought he would need six months to find another position. But he quickly found himself in another management position, as president and chief operating officer of South San Francisco-based Eos Biotechnology.

Williamson’s soft landing shows how people with the right skills can still find jobs in biotechnology, even when the markets are shaky. Part of his success stems from his willingness to take a risk. “At the senior executive level there aren’t a lot of people willing to do early biotech work,” he says.

Accepting risk means being “comfortable with

ambiguity and uncertainty”, he says. Avoiding recklessness means you have to do your homework.

When considering joining a company, “pretend you’re a venture capitalist”, he says. Check out its intellectual property, find out how much cash is on hand and who the primary investors are, and ensure that product development is rapid and mature. When considering a public company, read analysts’ reports before deciding.

For risk-averse biotech neophytes, a good strategy might be to join a well-established company with a good track record. When you get experience, it should be possible to move to a newer company, perhaps in a more senior position. **P.S.**

Rob Williamson (above) found another position easily when his old company folded. The abundance of biotech companies such as Genzyme (left) makes it easy for others in a similar position.

Domann and other recruiters agree that any move — from region to region, from company to company within a region, or from academia to industry — carries a risk. “People may want to take risks if they can have ownership,” he says.

Such risks don’t always pay off, and talented risk-takers can find themselves scrambling for a tenure-track academic position or a lower-level job in pharma, says Young. This can happen when “people are on their second or third biotech and realize that their stock options are again worthless”, says Domann. And sometimes even those who have had a successful career in mainstream industry decide to move back to academia (see “Academia to industry and back”). ■

Paul Smaglik is editor of *NatureJobs*; Adam Smith is editor of *Nature Reviews Drug Discovery*.